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PRINCIPAL INVESTIGATOR: Kenneth P. Nephew, Ph.D.
Meiyun Fan, Ph.D.
Teresa Craft
Annie Park

CONTRACTING ORGANIZATION: Indiana University
Indianapolis, IN 46202-5167

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6. AUTHOR(S)
Kenneth P. Nephew, Ph.D.
Meiyun Fan, Ph.D.
Teresa Craft
Annie Park

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Indiana University
Indianapolis, IN 46202-5167

E-Mail: knephew@indiana.edu

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Estrogens play important roles in both the onset and malignant progression of breast cancer. The content of estrogen receptors in breast tumors is a valuable predictor of whether a patient will respond to therapy with antiestrogens, such as tamoxifen and fulvestrant (ICI 182,780). Expression and activity of ER can be lost or impaired in antiestrogen-resistant breast cancer. The proposed studies are designed to test the overall hypothesis that the ubiquitin-like NEDD8 protein modification pathway represses estrogen action by facilitating degradation of ER protein. Perturbation of this pathway may prove instrumental in breast tumor progression; alternatively, activation of this pathway may prove to be a valid target for novel therapeutics. This study on mechanisms that regulate ER levels and activity are highly relevant to the development and progression breast cancer, including tumor progression to states of hormone independence and antiestrogen resistance. Thus, understanding how the estrogen receptor is regulated is an area of research critical to understanding the tissue selective pharmacology of estrogens. In addition, tamoxifen and other selective estrogen receptor modulators target the estrogen receptor, and this study is of the utmost relevance to those important therapies.

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INTRODUCTION

Estrogen regulates diverse biological processes through estrogen receptors (ERα and ERβ) (1). Receptor levels and dynamics have a profound influence on target tissue responsiveness and sensitivity to estrogen, and receptor turnover rates provide estrogen target cells with the capacity for rapid regulation of receptor levels and thus dynamic hormone responses (2-5). Furthermore, several experimental results have recently demonstrated that receptor degradation is a key component of the response of cancer cells, including breast cancer cells, to antiestrogen therapy (6, 7). In advanced stage breast cancers, estrogen receptor expression and activity can be lost or impaired, and the tumors are often resistant to endocrine therapies, such as the steroidal antiestrogens, ICI 182,780 and ICI 164,384 (6, 7). Our findings during the funding period have raised the intriguing possibility for a role of ubiquitin and ubiquitin-like pathways, including the NEDD8 pathway, in ERα ubiquitination and degradation and suggest that disruptions in such pathways may contribute to the development of antiestrogen-resistance in human breast cancer. The overall hypothesis that ubiquitin protein modification pathways repress estrogen action by facilitating degradation of ER protein was tested. Our experimental results suggest that perturbation of this pathway may prove instrumental in breast tumor progression; alternatively, activation of ubiquitin protein modification pathways may prove to be valid targets for novel therapeutics.

BODY

Task 1 was to determine the effect of Uba3 on breast cancer cell proliferation. We attempted to generate breast cancer cell lines stably expressing the dominant negative Uba3 (C216S), a mutant that we had used previously to block the NEDD8 pathway (8). However, blocking this pathway in MCF7 breast cancer cells was lethal and the cells died. We then attempted to an inducible promoter to control C216S expression levels, but these efforts were similarly unsuccessful. We conclude that the NEDD8 is essential for cell survival.

To further address this task, we generated a breast cancer cell line stably expressing a dominant negative Ubc12. The results of this investigation are described in Fan et al. (8 and manuscript in appendix), and some of the key findings are highlighted here. We established the stable cell line MCF7/ Ubc12C111S, which contains an impaired NEDD8 pathway and examined the effect of the antiestrogen on ERα degradation in these cells. Expression of Ubc12C111S inhibited ICI 182,780-induced ERα down-regulation (Fig. 5A). We then examined the growth inhibitory effect of ICI 182,780 in MCF/C111S cells. No significant difference was observed in basal cell proliferation rates between MCF7/C111S and MCF7/Vec cells in hormone-free medium (data not shown). Treatment with the antiestrogen inhibited basal cell growth of MCF7 and MCF7/Vec cells (Fig. 6A). In contrast, MCF7/C111S cells were partially resistant to ICI 182,780 (Fig. 6A, Left panel). Dose-response analysis showed that MCF7/C111S cells were resistant to a broad range of ICI 182,780 concentrations (Fig. 6A, Right panel). On the other hand, estradiol-induced proliferation of MCF7/C111S and control cells was similar (2-fold increase in cell number over a 6-day treatment period; data not shown). The effect of 4-OHT on MCF7/C111S and MCF7/Vec cell proliferation was examined in a time- and dose-response analysis. The response of the cell lines to 4-OHT was similar (Fig. 6B), suggesting that Ubc12C111S expression did not confer cells resistance to growth inhibitory effect of antiestrogens in general. These results suggest that the expression of Ubc12C111S conferred resistance of MCF7 cells to the growth inhibitory effects of ICI 182,780, but disrupting the NEDD8 pathway had no effect on the mitogenic response of MCF7 breast cancer cells to estradiol or the growth inhibitory effects of 4-OHT. Task one has been completed.

The second task of the project was to determine the molecular mechanisms of ERα corepression by the NEDD8 pathway. Toward this goal, we constructed Uba3 deletion constructs lacking one or both of the presumptive nuclear receptor interacting motifs (the NR boxes). GST-pulldown assays were conducted to determine which receptor domains mediate the interactions between ERα with Uba3. We were unable to detect direct interaction of the deletion mutant constructs with estrogen receptor (data not shown), suggesting that the NR boxes are essential for Uba3-ER interaction. However, this could also be due to important changes in protein conformation due to the removal of amino acid sequences. Thus, we took an alternative approach and generated point mutations within the NR boxes and then proposed to examine direct interactions of the mutant proteins with ER. Constructs were made and sequenced. However, we were unable to express proteins from the new constructs, for reasons that are unclear at this time. We speculate that perhaps the mutations made the protein unstable. Nonetheless, although mostly negative results were obtained, Task 2 has been completed.

Task 3 was to determine if ERα and ERα function is modified by APP-BP1 and Ubc12 and an NEDD8 target protein. First, we took a direct approach and determined if ERα is an NEDD8 target protein using co-transfection experiments, co-immunoprecipitation assays and Western blot analysis and looked for NEDD8-ER conjugates. We included various other components of the NEDD8 pathway, including co-transfecting Uba3, APPBP1, Ubc12 and various Cullin family members. We were unable to detect neddylated receptor (data not shown); therefore, we concluded that ER is not a direct substrate for modification by NEDD8. Next, we tested the hypothesis that the neddylation pathway may act to restrict

ERα activity by indirectly modulating receptor degradation. The results of this investigation are described in the manuscript in the appendix (8), and some of the key findings are highlighted below.

Coexpression of Uba3 decreased ERα protein level (Fig. 1A), and treatment with MG132, a specific proteasome inhibitor, blocked Uba3-stimulated down-regulation of ERα (Fig. 1B), confirming that the Uba3-induced ERα degradation is through the 26S proteasome. Overexpression of APP-BP1 or Ubc12 had no significant effect on ERα protein levels (data not shown), a result consistent with our previous observation that Uba3 is the limiting factor in neddylation-associated inhibition of ERα transcriptional activity (25). Next, to test the hypothesis that the neddylation pathway is required for ligand-mediated degradation of ERα, we used the dominant negative mutant of Ubc12, Ubc12C111S. Treatment of ERα transfected HeLa cells with estradiol resulted in a time-dependent decrease in ERα protein levels (Fig. 2A). In contrast, the effects of estradiol on receptor levels were less dramatic in cells expressing Ubc12C111S (Fig. 2A). Consistent with this observation, Uba3C216S, a dominant negative mutant of Uba3, also inhibited estradiol-induced ERα down regulation (Fig. 2B). Addition of the proteasome inhibitor MG132 prior to estradiol treatment completely abolished ligand-induced down-regulation of ERα (Fig. 2B), confirming that ERα undergoes proteasome-dependent degradation in response to estradiol. Collectively, these results demonstrate that a functional NEDD8 pathway is required for efficient, ligand-induced, proteasome-mediated degradation of ERα. Having established a role for the NEDD8 pathway in ERα down-regulation, we examined the effect of NEDD8 on receptor ubiquitination. Expression of dominant negative Ubc12C111S or Uba3C216S markedly decreased ERα ubiquitination in either the absence (Fig. 3, left panel) or presence of estradiol and MG132 (Fig. 3, right panel), compared to cells transfected with control vector or wild type Ubc12 or Uba3. These results suggest that a functional neddylation pathway is required for the efficient ubiquitination of ERα.

Having completed task 3, we continued to perform further investigations into the roles of ubiquitin-like pathway NEDD8 in the responses to estradiol and antiestrogens (deemed a logical extension of the SOW and within the scope of the fundamental questions underlying the SOW). Thus, the role of the ubiquitin-proteasome pathway in ERα-mediated transcriptional responses in breast cancer cells was investigated. Genetic and pharmacologic approaches were utilized to disrupt ERα ubiquitination, proteasome-mediated proteolysis and thus ERα degradation, including a dominant negative mutant of the NEDD8 conjugation enzyme (Ubc12C111S), the 20S proteasome inhibitor MG132, a ubiquitin mutant with all of its lysines mutated to arginine (UbK0), and the partial agonist/antagonist tamoxifen. To determine the effect of blocking ERα degradation on estradiol-induced transcriptional responses, estrogen receptor-responsive reporter assays and expression of endogenous ER-target genes in MCF7 human breast cancer cells were utilized. The results of this study are described in *Fan et al.* (9; manuscript in appendix), and some of the key findings are highlighted below.

We show that proteasomal degradation is not essential for transcriptional activity of ERα and suggest that the ubiquitin-proteasome system functions to limit estradiol-induced transcriptional output. The results demonstrate that blocking polyubiquitination of ERα stabilizes the receptor, resulting in the prolonged expression of ERα-responsive genes (Fig.1B,C). Inhibiting the proteasome enhanced ERα transcriptional activity in MCF7 human breast cancer cells (Fig. 5A,B), indicating that ERα degradation plays a key role in limiting estradiol-induced transcriptional responses in these cells. The results further suggest that in cells containing low levels of ERα, proteasome-mediated receptor degradation plays a role in limiting estradiol-induced transcriptional responsiveness (Figure 1B). While blocking ERα degradation increased the magnitude of estradiol-induced gene transcription, no effect on hormone sensitivity was observed (Fig. 2). However, inhibiting the proteasome increased both the magnitude and duration of estradiol-induced expression of an ERα-target gene in breast cancer cells (Fig. 5A). Overall, the data support the hypothesis that proteasome-mediated degradation of ERα serves as a means to limit the duration of estradiol signaling in receptor positive breast cancer cells. The important implication of this study is that the estradiol-induced transcriptional response is limited by receptor degradation through the ubiquitin-proteasome system, and defects in proteasome-mediated degradation of ERα could lead to an enhanced cellular response to estradiol in breast cancer cells.

Abnormal expression of ERα has long been associated with both the initiation and progression of breast cancer (10). An increase in the number of ERα-positive cells, as well as increased individual cell ERα content, have frequently been detected in malignant breast tumors (11). Furthermore, increased ERα content has been shown to augment the magnitude of estrogen-stimulated gene expression, providing a growth advantage to breast cancer cells (2, 8, 9,12). Collectively, these observations indicate that alterations in ERα degradation pathways may contribute to deregulation of ERα, perhaps leading to enhanced estrogen action in breast tumors.

We (described above) and others have clearly shown degradation of unliganded ERα is mediated by the ubiquitin-proteasome pathway, regulation of this pathway, at the molecular level, remains unclear. One potential mechanism involves CHIP, the carboxyl terminus of Hsc70-interacting protein, previously shown to target Hsp90 interacting proteins for ubiquitination and proteasomal degradation. We investigated a role for CHIP in degradation of unliganded ERα (13;

manuscript attached). In HeLa cells transfected with ERα and CHIP, ERα is downregulated through a ubiquitination dependent pathway, while ERα-mediated gene transcription decreased (Fig. 1 and Fig 2A). In contrast, siRNA inhibition of CHIP expression resulted in increased ERα accumulation and reporter gene transactivation (Fig 1B and Fig 2B). Transfection of mutant CHIP constructs demonstrated that both the U-box (containing ubiquitin ligase activity) and the tetratricopeptide repeat (TPR, essential for chaperone binding) CHIP domains are required for CHIP-mediated ERα downregulation (Fig 3). In addition, coimmunoprecipitation assays demonstrated that ERα and CHIP associate through the CHIP TPR domain (Fig 3). In ERα-positive breast cancer MCF7 cells, CHIP overexpression resulted in decreased levels of endogenous ERα protein and attenuation of ERα-mediated gene expression (Fig 4 and Fig 5). Furthermore, ERα-CHIP interaction was induced by the Hsp90 inhibitor geldanamycin (GA), resulting in enhanced ER-alpha degradation; this GA effect was further enhanced by CHIP overexpression, but was abolished by CHIP-siRNA (Fig 6-7). Finally, ERα dissociation from CHIP by various ERα ligands, including estradiol, tamoxifen, and ICI 182,780 interrupted CHIP-mediated ERα degradation (Fig 8). These results demonstrate a role for CHIP in both basal and GA-induced ERα degradation. Furthermore, based on our observations that CHIP promotes ERα degradation and attenuates receptor-mediated gene transcription, we suggest that CHIP, by modulating ERα stability, contributes to the regulation of functional receptor levels, and thus hormone responsiveness, in estrogen target cells. Thus, based on our results, the chaperone/CHIP pathway, by regulating ERα, levels, likely contributes to the development/progression of breast cancer. We believe that such a possible role for CHIP in breast cancer merits further examination.

In addition, during the funding period, we contributed to collaborative projects on the regulation of ER target genes in breast cancer, resulting in a co-authored publication (14; manuscript in appendix).

KEY RESEARCH ACCOMPLISHMENTS

- Showed that the neddylation pathway is required for ligand-mediated degradation of ERα
- Discovered that the NEDD8 pathway is required for efficient ubiquitination of ERα
- Established that disrupting the NEDD8 pathway confers antiestrogen resistance in breast cancer cells
- Provided evidence that allowed us to speculate that disruptions in the NEDD8 pathway may provide a mechanism by which breast cancer cells acquire ICI 182,780 resistance while retaining expression of ERα.
- Showed that that ERα degradation plays a key role in limiting estradiol-induced transcriptional responses in MCF7 human breast cancer cells.
- Demonstrated that inhibiting the proteasome increased estradiol-induced expression of an ERα-target gene in breast cancer cells.
- Determined that proteasomal degradation is not essential for transcriptional activity of ERα and that the ubiquitin-proteasome system appears to function to limit estradiol-induced transcriptional output.
- Provided evidence to suggest that defects in proteasome-mediated degradation of ERα could lead to an enhanced cellular response to estradiol in breast cancer cells.
- Demonstrated that CHIP promotes ERα degradation and attenuates receptor-mediated gene transcription.
- Provided evidence to suggest that CHIP, by modulating ERα stability, contributes to the regulation of functional receptor levels, and thus hormone responsiveness, in estrogen target cells.
- Provided evidence to suggest the chaperone/CHIP pathway, by regulating ERα, levels, may contribute to the development/progression of breast cancer.

REPORTABLE OUTCOMES

Manuscripts

1. *Fan M, Bigsby RM, **Nephew KP** 2003 The NEDD8 pathway is required for proteasome mediated degradation of human estrogen receptor-α and essential for the antiproliferation activity of ICI 182,780 in ER-positive breast cancer cells Mol Endocrinol 17:356-365 (cover article)
2. *Fan M, Nakshatri H, **Nephew KP**. 2004. Inhibiting proteasomal proteolysis sustains estrogen receptor-alpha activation. Mol Endocrinol 18:2603-2615
3. *Fan M, Park A, **Nephew KP**. Interactions between estrogen receptor and the COOH terminus of the Hsp70-interacting protein (CHIP) (Mol Endocrinol under revision)
4. *Leu YW, Yan PS, Fan W, Jin VX, Liu CJ, Curran EM, Welshons WV, Wei HS, Davuluri RV, Plass C, **Nephew KP,** Huang TH-M. 2004. Loss of estrogen signaling triggers epigenetic silencing of downstream targets Cancer Res 64:8184-8192 (cover article).

*This DOD award is acknowledged in these publications.

Presentations

1. Fan M, Long X, Bailey JA, Reed CA, Gize EA, Osborne E, Kirk EA, Bigsby RM, **Nephew KP** The activating enzyme of NEDD8 inhibits steroid receptor function. Keystone Symposium on Nuclear Receptor Superfamily, April, 2002
2. Fan M, Bigsby RM, **Nephew KP** 2002 Role for the neddylation pathway in estrogen receptor ubiquitination and degradation. 84th Annual Meeting of the Endocrine Society, June 19-22, San Francisco, CA (platform talk)
3. Fan M, Bigsby RM, **Nephew KP** 2002 Role for the neddylation pathway in estrogen receptor ubiquitination and degradation. Midwest Regional Molecular Endocrinology Conference, Indiana University, Bloomington, IN (platform talk)
4. Fan M, Nakshatri H, **Nephew KP** The role of proteasome-mediated estrogen receptor-α (ER) degradation in estrogen responsiveness 94th annual meeting of the American Association for Cancer Research, Toronto, Ontario, Canada (poster/discussion).
5. Fan M, Nakshatri H, **Nephew KP** 2003 The role of proteasome-mediated degradation of estrogen receptor-α in estrogen-induced transcriptional response. Elwood Jensen Symposium on Nuclear Receptors and Endocrine Disorders. University of Cincinnati, December 5-7 (platform talk).
6. Fan M, Park A, **Nephew KP** 2005 CHIP (Carboxyl Terminus of Hsc70-Interacting Protein) promotes basal and geldanamycin-induced degradation of estrogen receptor-α. 87th annual Meeting of The Endocrine Society, San Diego, CA (platform talk).

CONCLUSIONS

The antiestrogen ICI 182,780 is a drug is used as a second-line endocrine agent in patients who have developed tamoxifen-resistant breast cancer. Despite its potent antitumor effects, the drug does not circumvent the development of antiestrogen resistance (15-17). Moreover, the fact that most tumors acquiring ICI 182,780 resistance do so while retaining expression of ERα and estrogen responsiveness (18-20), suggests that administration of the antiestrogen may possibly lead to the selection of tumor cells defective in ERα down-regulation pathway(s), which in turn may confer a proliferative advantage in either the presence or absence of estrogens. In this context, mechanism underlying persistent expression of ERα in tumors with acquired resistance, such as disruptions in the NEDD8, CHIP or other ubiquitin or ubiquitin-associated/protein receptor degradation pathways, may thus present an important therapeutic target for future drug intervention.

For the "so what section" (evaluates the knowledge as a scientific or medical product to also be included in the conclusion of this report), the loss of ERα degradation pathway(s) may provide a mechanism by which breast cancer cells acquire antiestrogen resistance while retaining expression of ERα. Pathways that utilize the ubiquitin-proteasome system could serve as a therapeutic targets for breast cancer.

In summary, all three tasks have been completed. .

List of personnel receiving pay from the research effort: Kenneth P. Nephew, Ph.D., Principal Investigator; Meiyun Fan, Ph.D., Postdoctoral Fellow; Teresa Craft, M.S., Research Associate, Annie Park, B.S., Research Associate, Xinghua Long, Graduate Student.

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8. Fan M, Bigsby RM, **Nephew KP** 2003 The NEDD8 pathway is required for proteasome mediated degradation of human estrogen receptor-α and essential for the antiproliferation activity of ICI 182,780 in ER-positive breast cancer cells Mol Endocrinol 17:356-365

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APPENDICES

Reprints:

1. Fan M, Bigsby RM, **Nephew KP** 2003 The NEDD8 pathway is required for proteasome mediated degradation of human estrogen receptor-α and essential for the antiproliferation activity of ICI 182,780 in ER-positive breast cancer cells Mol Endocrinol 17:356-365 (cover article)
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APPENDIX 1

The NEDD8 Pathway Is Required for Proteasome-Mediated Degradation of Human Estrogen Receptor (ER)-α and Essential for the Antiproliferative Activity of ICI 182,780 in ERα-Positive Breast Cancer Cells

MEIYUN FAN, ROBERT M. BIGSBY, AND KENNETH P. NEPHEW

Medical Sciences (M.F., K.P.N.), Indiana University School of Medicine, Bloomington, Indiana 47405; and Departments of Obstetrics & Gynecology (R.M.B., K.P.N.) and of Cellular and Integrative Physiology (R.M.B., K.P.N.), Indiana University Cancer Center (R.M.B., K.P.N.), Indiana University School of Medicine, Indianapolis, Indiana 46202

Steroid hormone receptors, including estrogen receptor-α (ERα), are ligand-activated transcription factors, and hormone binding leads to depletion of receptor levels via preteasome-mediated degradation. NEDD8 (neural precursor cell-expressed developmentally down-regulated) is an ubiquitin-like protein essential for protein processing and cell cycle progression. We recently demonstrated that ubiquitin-activating enzyme (Uba)3, the catalytic subunit of the NEDD8-activating enzyme, inhibits ERα transcriptional activity. Here we report that Uba3-mediated inhibition of ERα transactivation function is due to increased receptor protein turnover. Coexpression of Uba3 with ERα increased receptor degradation by the 26S proteasome. Inhibition of NEDD8 activation and conjugation diminished polyubiquitination of ERα and blocked proteasome-mediated degradation of receptor protein. The antiestrogen ICI 182,780 is known to induce ER degradation. In human MCF7 breast cancer cells modified to contain a disrupted NEDD8 pathway, ICI 182,780 degradation of ERα was impaired, and the antiestrogen was ineffective at inhibiting cell proliferation. This study provides the first evidence linking nuclear receptor degradation with the NEDD8 pathway and the ubiquitin-proteasome system, suggesting that the two pathways can act together to modulate ERα turnover and cellular responses to estrogens. Based on our observation that an intact NEDD8 pathway is essential for the antiproliferation activity of the ICI 182,780 in ERα positive breast cancer cells, we propose that disruptions in the NEDD8 pathway provide a mechanism by which breast cancer cells acquire antiestrogen resistance while retaining expression of ERα. (*Molecular Endocrinology* 17: 356–365, 2003)

ESTROGEN REGULATES DIVERSE biological processes through estrogen receptors (ERα and ERβ) (1). Receptor levels and dynamics have a profound influence on target tissue responsiveness and sensitivity to estrogen (2). ERα is a short-lived protein with a half-life of about 4 h, which is reduced to 3 h by 17β-estradiol (estradiol), and to less than 1 h by the steroidal antiestrogens, ICI 182,780 and ICI 164,384 (3, 4). Receptor turnover rates provide estrogen target cells with the capacity for rapid regulation of receptor levels and thus dynamic hormone responses. An attenuated transcriptional response has been associated with down-regulation of ERα, and receptor up-regulation has been shown to enhance the cellular response to estrogen (2). Nonetheless, mechanisms governing ERα protein levels remain poorly understood.

It has recently been shown that degradation of ERα and other members of the nuclear receptor superfamily occurs through the ubiquitin-proteasome pathway (5). Ubiquitination is a multistep process involving the action of a ubiquitin-activating enzyme (E1 or Uba), a ubiquitin conjugation enzyme (E2 or Ubc), and a ubiquitin ligase (E3) (6). Because the high specificity for target proteins is primarily conferred by E3, regulation of E3 activity may play a crucial role in governing protein degradation *in vivo*. A large number of E3s are cullin-based ubiquitin ligases (7), including SCF (Skp1/Cul1/F-box/ROC1) and VCB (von Hippel-Lindau-Cul2/elongin B/elongin C) complexes. One important level of regulation of these cullin-based ubiquitin ligases involves modification of the cullin subunit with NEDD8, an ubiquitin-like protein (7).

NEDD8 conjugation (neddylation) resembles ubiquitination and involves the action of amyloid precursor protein-binding protein (APP-BP1)/Uba3, a heterodimeric E1-like enzyme, and Ubc12, an E2-like enzyme (8).

Abbreviations: APP-BP1, Amyloid precursor protein-binding protein; AR, androgen receptor; csFBS, charcoal-stripped FBS; E2, ubiquitin conjugation enzyme; E3, ubiquitin ligase; ER, estrogen receptor; estradiol, 17β-estradiol; FBS, fetal bovine serum; HA, hemagglutinin; GAPDH, glyceraldehyde phosphate dehydrogenase; GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NEDD8, neural precursor cell-expressed developmentally down-regulated; 4-OHT, 4-hydroxytamoxifen; PR, progesterone receptor; Uba, ubiquitin-activating enzyme; Ubc, ubiquitin-conjugation enzyme.

Whether a ligase is required for neddylation is unknown. To date, the only known substrates of NEDD8 are cullin family members (9, 10). Cullin neddylation is conserved and plays an important regulatory role for cullin-based E3 activity in yeast, plant, and mammalian cells (7, 11–13). Interrupting NEDD8 modification of cullins in mammalian cells has been shown to block ubiquitination of certain proteins involved in different cellular functions, including p27, IκBα, HIFα, and NFκB precursor p105 (14–19). Recent studies have revealed that cullin neddylation is a tightly controlled dynamic process (20–24), and the effect of neddylation on protein polyubiquitination appears to be specific (17, 18).

We recently identified the NEDD8 activating enzyme, Uba3 as an ER-interacting protein and inhibitor of transactivation by steroid nuclear receptors (25). We further demonstrated that an intact neddylation pathway is required for Uba3-mediated inhibition of ER transcriptional activity (25). Taken together with recent reports linking the ubiquitin and NEDD8 pathways (7), our findings raise the intriguing possibility for a role of neddylation in ERα ubiquitination and degradation. Here we show that Uba3 enhances ERα degradation by the 26S proteasome, and expression of dominant-negative mutants of Uba3 or Ubc12 impaired ERα ubiquitination and ligand-induced ERα degradation. Blocking the neddylation pathway with the dominant-negative Ubc in ERα-positive human breast cancer cells inhibited both receptor degradation and the growth inhibitory effect of the antiestrogen ICI 182,780 (known clinically as Faslodex or Fulvestrant). Collectively, these data show that the NEDD8 pathway plays an essential role in ubiquitination and proteasomal degradation of ERα and indicate that disruptions in the pathway may contribute to the development of antiestrogen resistance in human breast cancer.

RESULTS

Uba3 Enhances Proteasomal Degradation of ERα

To test the hypothesis that the neddylation pathway restricts ERα activity by modulating receptor degradation, we transfected HeLa cells with ERα, alone or in combination with an expression vector for Uba3, APP-BP1, or Ubc12, or with an empty vector (pcDNA3.1, Invitrogen, Carlsbad, CA); a green fluorescence protein (GFP) expression vector was cotransfected to serve as a means of normalizing transfection efficiency and sample preparations. Steady-state levels of ERα protein were determined by Western blot analysis. Coexpression of Uba3 decreased ERα protein level but had no effect on GFP expression (Fig. 1A). Treatment of the transfected HeLa cells with MG132, a specific proteasome inhibitor, blocked Uba3-stimulated down-regulation of ERα (Fig. 1B), confirming that the Uba3-induced ERα degradation is through the 26S proteasome. Overexpression of APP-BP1 or Ubc12 had no significant effect on ERα protein levels (data not shown), a result consistent with our previous observation that Uba3 is the limiting factor in neddylation-associated inhibition of ERα transcriptional activity (25).

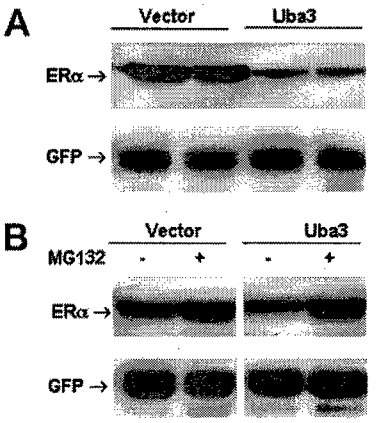

Fig. 1. Uba3 Enhances Proteasomal Degradation of ERα

A, Coexpression of Uba3 decreases ERα protein level in transfected HeLa cells. HeLa cells were transfected with pSG5-ER and pcDNA-Uba3 or pcDNA vector. Whole cell extracts were prepared 24 h post transfection and analyzed by Western blotting to determine ERα protein level. B, Proteasome inhibitor MG132 restores expression level of ERα in cells transfected with Uba3. Transfected HeLa cells (same as in A) were treated with 20 μM MG132 for 6 h before protein extracts and ERα level analysis. GFP was used as an internal control to correct for transfection efficiency and SDS-PAGE loading. Representative results of three independent experiments are shown.

The Neddylation Pathway Is Required for Ligand-Mediated Degradation of ERα

Estradiol stimulates ERα degradation through the ubiquitin-proteasome pathway (26–30). Having established a role for Uba3 in this process, it was important to assess whether neddylation pathway is required for ligand-induced degradation of ERα. To address this issue, we used a dominant-negative mutant of Ubc12 (Ubc12C111S). Due to a single Cys-to-Ser substitution at the active Cys residue, Ubc12C111S forms a stable complex with NEDD8, resulting in sequestration of NEDD8 and inhibition of subsequent NEDD8 conjugation (31, 32). Dominant-negative inhibition of NEDD8 conjugation by Ubc12C111S has been shown to impair efficient ubiquitination and protein degradation (14, 15, 17, 18). Treatment of ERα-transfected HeLa cells with estradiol resulted in a time-dependent decrease in ERα protein levels; receptor levels were reduced by 80% at 6–8 h. (Fig. 2A). In contrast, the effects of estradiol on receptor levels were less dramatic in cells expressing Ubc12C111S, producing a reduction of only 40% by 6–8 h (Fig. 2A). Consistent with this observation, Uba3C216S, a dominant-negative mutant of Uba3 (31, 32), also inhibited estradiol-induced ERα down-regulation (Fig. 2B). Addition of the proteasome inhibitor MG132 before estradiol treatment completely abolished ligand-induced down-regulation of ERα (Fig. 2B), con-

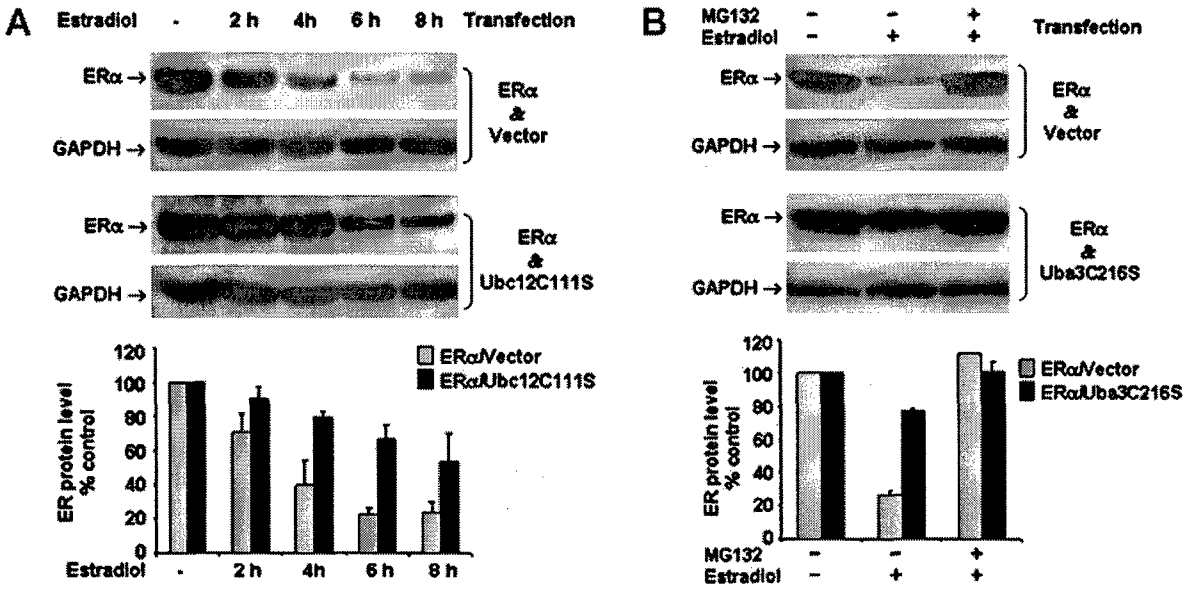

Fig. 2. Expression of Ubc12C111S or Uba3C216S Inhibits Ligand-Induced ERα Degradation

A, HeLa cells were transfected with pSG5-ER and pcDNA vector (*upper panel*) or pcDNA-Ubc12C111S (*lower panel*). Twenty-four hours after transfection, cells were treated with 100 nM estradiol for the indicated times and analyzed for ERα protein level using Western blotting. Relative ERα levels in cells cotransfected with vector (*gray*) or Ubc12C111S (*black*) from two independent experiments are shown in corresponding histogram. B, HeLa cells were transfected with pSG5-ER and pcDNA vector (*upper panel*) or pcDNA-Uba3C216S (*lower panel*). Twenty-four hours after transfection, cells were treated with vehicle or 20 μM MG132 for 1 h followed by incubation with vehicle or 100 nM estradiol for 6 h, as indicated. ERα protein levels were analyzed by immunoblotting. Relative ERα levels in cells cotransfected with vector (*gray*) or Uba3C216S (*black*) from three independent experiments are shown in corresponding histogram. GAPDH was used as an internal control to correct SDS-PAGE loading.

firming that exogenous ERα in HeLa cells undergoes proteasome-dependent degradation in response to estradiol. Collectively, these results demonstrate that a functional NEDD8 pathway is required for efficient, ligand-induced, proteasome-mediated degradation of ERα.

The NEDD8 Pathway Is Required for Efficient Ubiquitination of ERα

Having established a role for Uba3 and Ubc12 in ERα down-regulation, it was important to examine the effect of NEDD8 on receptor ubiquitination. HeLa cells were cotransfected with ERα and hemagglutinin (HA)-tagged ubiquitin, along with wild-type Ubc12 or Uba3 or the corresponding mutant forms of these neddylation enzymes (Ubc12C111S or Uba3C216S). At 24 h post transfection, cells were treated with MG132 or vehicle, followed by estradiol treatment. Immunoprecipitation assays using an anti-ERα antibody were performed and the levels of ubiquitinated ERα in the precipitated immunocomplex were assessed by Western blotting with an anti-HA antibody. The polyubiquitinated ERα exhibited a ladder of higher molecular weight species on the blot membrane (Fig. 3). Expression of dominant-negative Ubc12C111S or Uba3C216S markedly decreased ERα ubiquitination in either the absence (Fig. 3, *left panel*) or presence of estradiol and MG132 (Fig. 3, *right panel*), compared with cells transfected with control vector or wild-type Ubc12 or Uba3. These results suggest that a functional neddylation pathway is required for the efficient ubiquitination of ERα.

ERα Protein Levels in MCF7 Breast Cancer Cell Lines Stably Expressing Dominant-Negative Ubc12C111S

MCF7 human breast cancer cells express high levels of ERα and proliferate in response to estrogen treatment (33, 34), providing a model to study endogenous ERα function. To further investigate the role of neddylation in ERα function under physiological relevant conditions, we transfected Ubc12C111S into MCF7 cells and established the stable cell line MCF7/C111S. As a control, MCF7/Vec (MCF7 cells stably transfected with empty vector) was also established. Expression of the Ubc12C11S mutant protein in MCF7/C111S cells was confirmed by Western blotting and, consistent with a previous report (31), the mutant was detected as 26- and 31-kDa proteins (Fig. 4, lanes 3–8). In the regular growth medium containing phenol red and 10% fetal bovine serum (FBS), the level of ERα in MCF7/Vec cells was very low; after 3 d of culture in hormone-free medium containing 3% dextran-coated charcoal-stripped FBS (cs-FBS) and no phenol red, ERα expression was dramatically increased (Fig. 4, lanes 1 and 2). The culture medium (regular growth medium *vs.* hormone-free me-

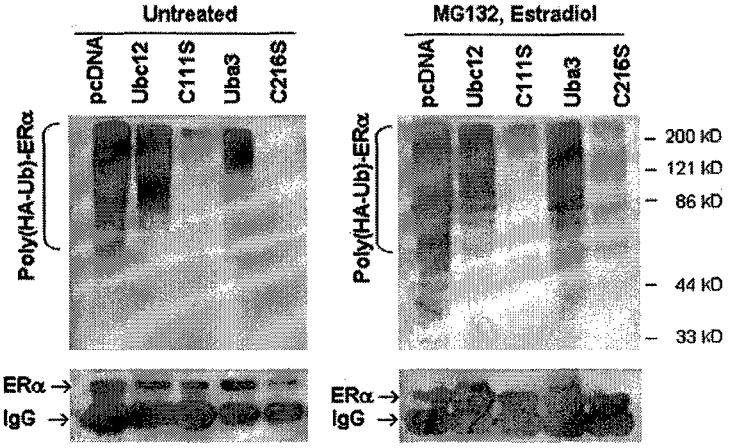

Fig. 3. An Intact NEDD8 Pathway Is Required for Efficient ERα Ubiquitination

HeLa cells were transfected with pSG5-ER, and pcDNA-HA-Ubiquitin, alone with indicated construct. Twenty-four hours after transfection, cells were either untreated (*left panel*) or treated with 20 μM MG132 for 1 h followed by 100 nM estradiol exposure for 3 h (*right panel*). Protein extracts were prepared and subjected to immunoprecipitation using anti-ERα antibody. Polyubiquitinated ERα was detected by Western blotting using anti-HA antibody, and was visualized as a ladder of higher molecular weight species on the blot. The blot was striped and reprobed by anti-ERα antibody to assess the amount of precipitated ERα (*lower panels*). The heavy chain of the anti-ERα IgG used for immunoprecipitation exhibits a 57-kDa band in the ERα blot. Representative results of three independent experiments are shown.

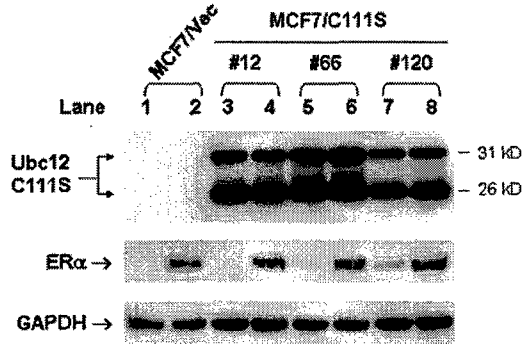

Fig. 4. The Expression of Ubc12C111S and ERα in Three Independent MCF7/C111S Clones

MCF7/C111S cells stably expressing mutant Ubc12C111S were maintained in growth medium (lanes 1, 3, 5, and 7) or hormone-free medium for 3 d (lanes 2, 4, 6, and 8) and analyzed by immunoblotting using anti-HA (*upper panel*) or anti-ERα (*lower panel*) antibodies, respectively. GAPDH was used as an internal control to correct for SDS-PAGE loading.

dium) showed no effect on the expression level of Ubc12C111S. In three MCF7/C111S clones, receptor levels varied among the clones and, when cultured in growth medium, detectable ERα was seen in two of the three clones (Fig. 4, lanes 5 and 7). When cultured in estrogen-free medium, however, ERα levels were high in all three clones (Fig. 4, lanes 4, 6, 8).

Ubc12C111S Inhibits ICI 182,780-Induced Down-Regulation of ERα

In contrast to estradiol, which down-regulates ERα in target tissues through both transcriptional and posttranslational mechanism (35, 36), the pure antiestrogen ICI 182,780 causes ERα protein degradation without affecting ERα mRNA levels (3, 36). Based on our observations that the NEDD8 pathway is essential for ERα degradation in transfected HeLa cells (Fig. 2), it was of interest to examine the effect of the antiestrogen on ERα degradation in MCF7/C111S cells. Cells were cultured in hormone-free medium for 3 d before ICI 182,780 treatment. Under this condition, comparable amounts of ERα were observed in MCF7/C111S and MCF7/Vec cells (compare 0-h lanes in Fig. 5A). Treatment with ICI 182,780 rapidly (by 1 h) decreased ERα levels in the MCF7/Vec cells; by 4 h post treatment, the levels of ERα were reduced by 95% (Fig. 5A). In the MCF7/C111S cells, the effects of ICI 182,780 on ERα levels were much less dramatic (Fig. 5A). Thus, although ER degradation was not completely inhibited by expression of the dominant-negative Ubc12C111S, these results confirm our observations using transient transfection in HeLa cells and further suggest that the NEDD8 pathway is required for efficient degradation of endogenous ERα. To examine the effect of another antiestrogen on ERα degradation in this system, cells were cultured in the presence of various doses of 4-hydroxytamoxifen (4-OHT) and ERα levels were examined. In both MCF7/Vec and MCF7/C111S cells, ERα levels remained unchanged or were slightly increased after treatment with 4-OHT (Fig. 5B). Stabilization of ERα by tamoxifen has been reported by others (30), perhaps due to inhibition of the basal rate of ER degradation by the antiestrogen.

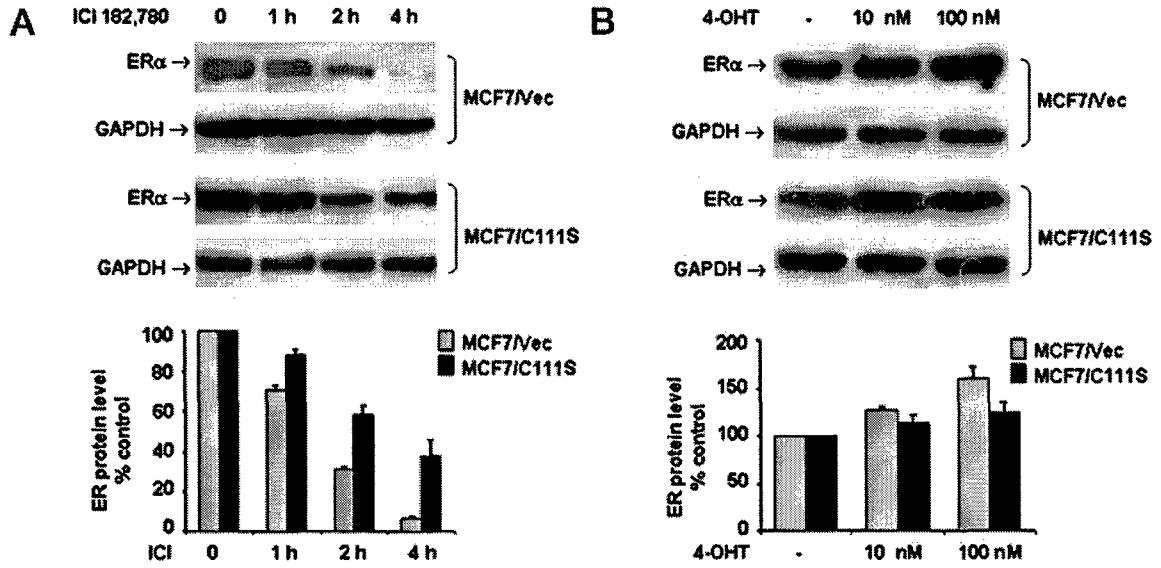

Fig. 5. ERα Degradation Is Impaired in MCF7/C111S Cells

A, ICI 182,780-induced ERα degradation is impaired in MCF7/C111S cells. MCF7/Vec (*upper panel*) and MCF7/C111S cells (*lower panel*) were cultured in hormone-free medium for 3 d and treated with 1 nM ICI 182,780 for the indicated times. B, 4-OHT does not cause ERα degradation in MCF7 cells. MCF7/Vec (*upper panel*) and MCF7/C111S cells (*lower panel*) were cultured in hormone-free medium for 3 d and treated with indicated doses of 4-OHT for 6 h. ERα protein levels were determined by Western blotting with anti-ERα antibody. The histogram shows the relative ERα levels after ICI 182,780 or 4-OHT treatment. Relative ERα levels in MCF7/vec (*gray*) from three independent experiments or MCF7/C111S (*black*) from three independent MCF7/C111S clones are shown in corresponding histogram. GAPDH was used as an internal control to correct SDS-PAGE loading.

Disrupting the NEDD8 Pathway Confers Antiestrogen Resistance in Breast Cancer Cells

Estradiol is mitogenic in MCF7 cells and stimulates cell proliferation through activation of ERα (37). The pure antiestrogen ICI 182,780, on the other hand, blocks ERα-mediated transactivation and induces ERα protein degradation, resulting in growth inhibition of breast cancer cells (38). Because expression of Ubc12C111S inhibited ICI 182,780-induced ERα down-regulation (Fig. 5A), we examined the growth inhibitory effect of ICI 182,780 in MCF7/C111S cells. No significant difference was observed in basal cell proliferation rates between MCF7/C111S and MCF7/Vec cells in hormone-free medium (data not shown). Treatment with the antiestrogen (1 nM) inhibited the basal cell growth of MCF7 and MCF7/Vec cells (Fig. 6A). In contrast, MCF7/C111S cells were partially resistant to ICI 182,780. Specifically, over an 8-d period, the antiestrogen inhibited the growth of control cells by 50% compared with 20–25% growth inhibition of the MCF7/C111S cells (Fig. 6A, *left panel*). Dose-response analysis showed that MCF7/C111S cells were resistant to a broad range (0.01–10 nM) of ICI 182,780 concentrations (Fig. 6A, *right panel*). On the other hand, estradiol-induced proliferation of MCF7/C11S and control cells was similar (2-fold increase in cell number over a 6-d treatment period; data not shown). The effect of 4-OHT on MCF7/C111S and MCF7/Vec cell proliferation was examined in a time- and dose-response analysis. The response of the cell lines to 4-OHT was similar (Fig. 6B), suggesting that Ubc12C111S expression did not confer cells resistance to growth inhibitory effect of antiestrogens in general. These results suggest that the expression of Ubc12C111S conferred resistance of MCF7 cells to the growth inhibitory effects of ICI 182,780, but disrupting the NEDD8 pathway had no effect on the mitogenic response of MCF7 breast cancer cells to estradiol or the growth inhibitory effects of 4-OHT.

DISCUSSION

ERα is a short-lived protein whose degradation is primarily mediated by the ubiquitin-proteasome pathway (26–30). The recently described ubiquitin-like pathways, including the NEDD8 and SUMO (small ubiquitin-like modifier) conjugation systems (39), have been implicated in nuclear receptor regulation (40–44) and the NEDD8 pathway has been shown to enhance protein polyubiquitination (12, 14–19, 45–47). Our previous investigation into the role of the NEDD8 pathway in nuclear hormone receptor regulation showed that Uba3, the catalytic subunit of the NEDD8 activating enzyme complex, interacts with ERα and inhibits receptor function (25). Here we report that Uba3-mediated inhibition of ERα transactivation is due to increased receptor turnover and that an intact neddylation pathway is essential for ERα ubiquitination and degradation. By impairing the NEDD8 path-

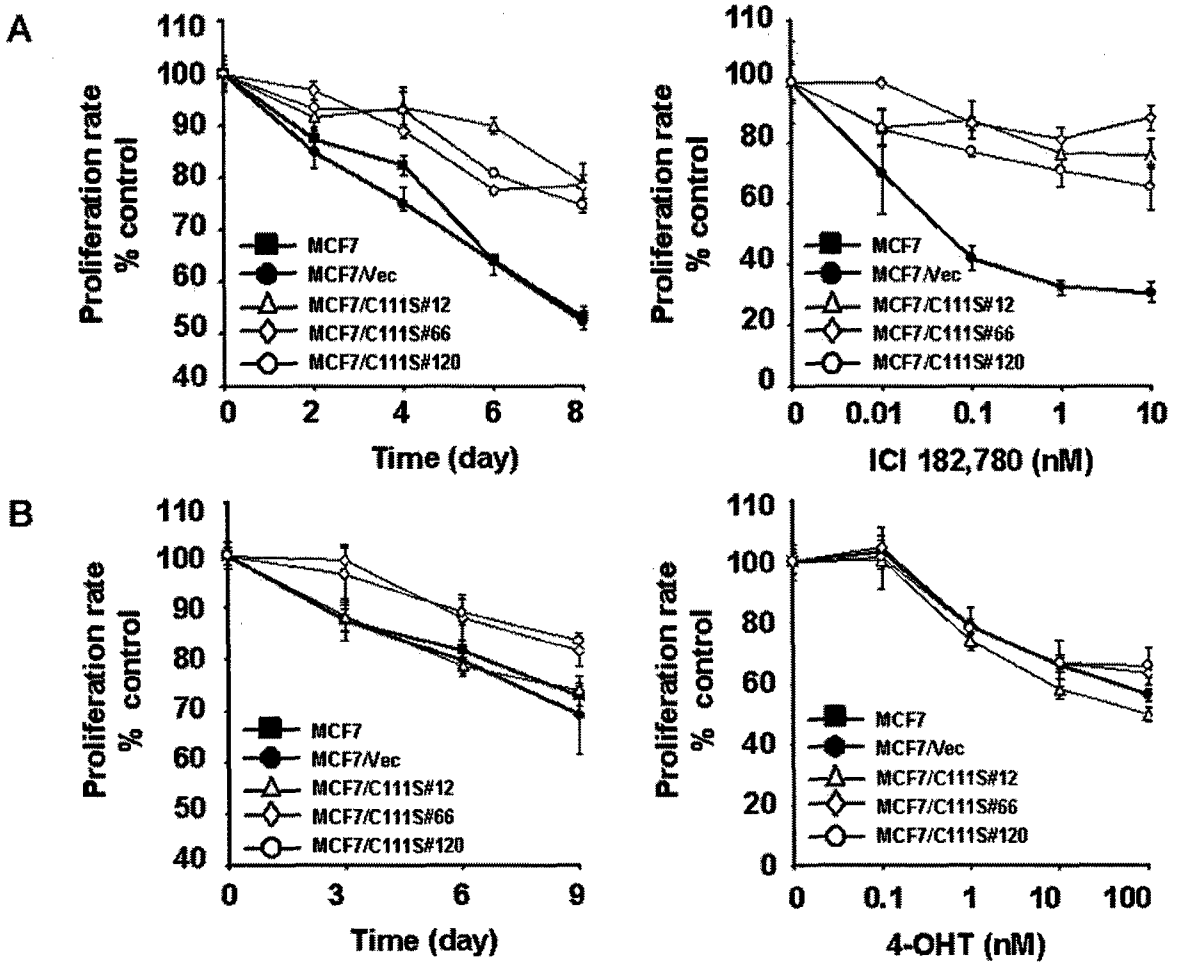

Fig. 6. Interruption of the NEDD8 Pathway Confers Resistance to ICI 182,780 in Human Breast Cancer Cells

A, Time- and dose-dependent growth inhibition of ICI 182,780. For time-response analysis, cells were treated with 1 nM ICI 182,780 and cell numbers were determined 0, 2, 4, 6, and 8 d after drug exposure. For dose-response assay, cells were treated with indicated doses of ICI 182,780 and cell numbers were determined on d 7. B, Time- and dose-dependent antiproliferative effect of 4-OHT. For time-response analysis, cells were treated with 10 nM 4-OHT and cell numbers were determined 0, 3, 6, and 9 d later. For the dose-response assay, cells were treated with indicated doses of 4-OHT and cell numbers were determined on d 7. For all assays, cells were cultured in hormone-free medium for 3 d before treatment and cell numbers were determined by MTT assay. Relative proliferation rate was expressed as percentage of cells grown in hormone-free medium. Each experiment was repeated three times in quadruplicate.

way in human MCF7 breast cancer cells, we demonstrated that the cells became resistant to the growth inhibitory effects of ICI 182,780. Thus, our data suggest that neddylation plays an important role in ERα degradation and we speculate that alterations in the NEDD8 pathway may provide a mechanism by which tumors can acquire antiestrogen resistance.

Several recent studies have focused on the role of the ubiquitin-proteasome pathway in nuclear receptor down-regulation (26–30). Enhancement of ERα ubiquitination by estradiol was first reported by Nirmala and Thampan (48), and Nawaz *et al.* (27) showed that a functional ubiquitin-proteasome system is required for ERα degradation. Both basal and ligand-induced ERα ubiquitination occurs at the nuclear matrix (49), but how ERα is targeted for ubiquitination has not been fully established. Previously, we had shown that Uba3 interacts directly with ER and that this interaction is augmented by estradiol (25). Here, we show that overexpression of Uba3 enhanced degradation of ERα and that disruption of Uba3 activity reduces estradiol-induced receptor degradation. Taken together, these data support a role for Uba3 in the regulation of basal as well as ligand-induced ERα turnover.

The present study is the first to link the NEDD8 pathway to ubiquitination of ERα. The exact mechanism connecting the two pathways, however, remains unclear. The only known substrates for direct neddylation are members of the cullin family (10). Some of the cullins have been identified as core subunits of specific ubiquitin ligase complexes (7). Mechanistically, conjugation of NEDD8 to cullins may up-regulate

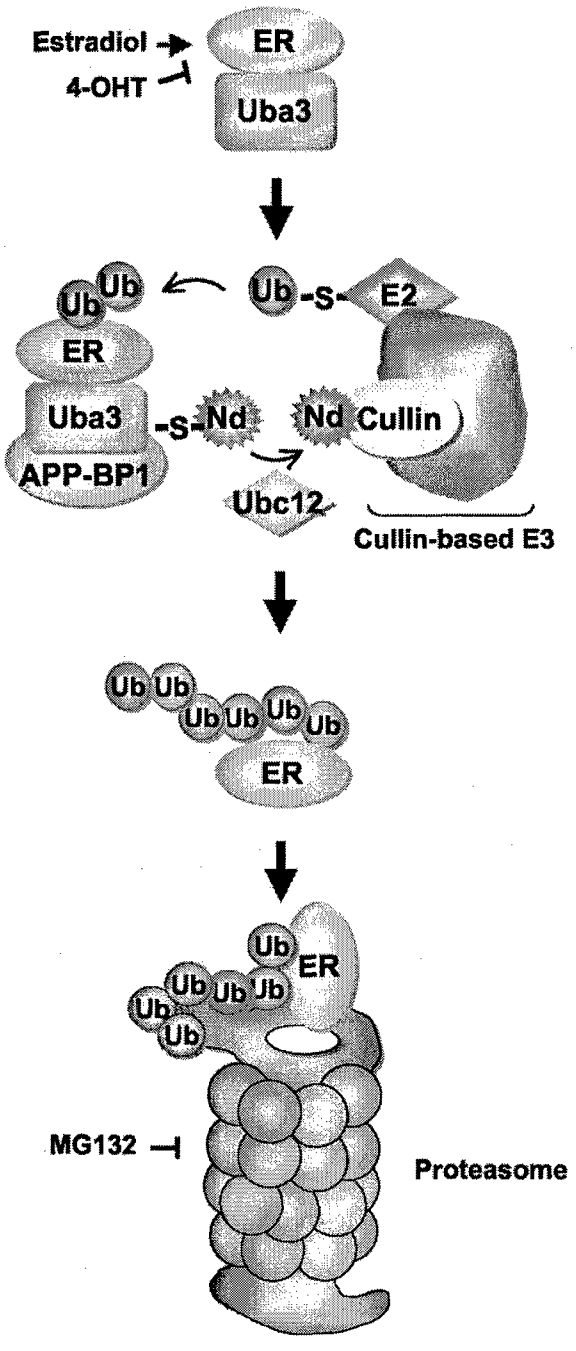

Fig. 7. Hypothetical Model Depicting the Role of Neddylation Pathway in Proteasome-Mediated Degradation of ERα

The physical interaction between Uba3 and ERα promotes the functional recruitment and activation of a cullin-based ubiquitin-protein ligase to augment receptor polyubiquitination. Uba3 and APP-BP1, the heterodimeric activating enzyme for NEDD8, and Ubc12, the NEDD8 conjugating enzyme, promote cullin NEDD8 modification of specific ubiquitin E3 ligases. Neddylated cullins enhance the formation and activity of the ubiquitin E2-E3 complex. The potency of ERα-Uba3 interaction appears to correlate with ERα turnover rate. In the absence of ligand, ERα interacts weakly with Uba3, resulting in basal ubiquitination and degradation of ERα; however, estradiol augments the ERα-Uba3 interaction to enhance ERα ubiquitination. On the other hand, 4-OHT interrupts the ERα-Uba3 interaction and stabilizes ERα, and MG132 blocks ERα degradation by inhibiting proteasome activity. APP-BP1, Amyloid precursor protein-binding protein; E2, ubiquitin conjugation enzyme; E3, ubiquitin protein ligase; estradiol, 17β-estradiol; Nd, neural precursor cell-expressed developmentally down-regulated (NEDD8); ↓ and ⊥, Stimulation and inhibition, respectively.

ubiquitin ligase activity of specific E3s by facilitating the formation of an ubiquitin E2-E3 complex (45). In this regard, the interaction between Uba3 and ERα could result in the functional recruitment and activation of a cullin-based ubiquitin-protein ligase, which, in turn, targets ERα for degradation by the ubiquitin-proteasome system. The hypothetical model depicting the role of neddylation pathway in proteasome-mediated degradation of ERα is shown in Fig. 7. Together with our previously reported data (25), these observations indicate that such targeted degradation of ERα leads to reduced hormonal responsiveness.

In addition to its effect on ERα, Uba3 inhibits the transactivation function of other steroid receptors, ERβ, androgen receptor (AR) and progesterone receptor (PR) (25). Others have reported that NEDD8 interacts with aryl hydrocarbon receptor and the interaction affects the transcriptional activity and stability of the receptor protein (40). Furthermore, the NEDD8 protein has been found to colocalize with AR (50). Together with the observations that turnover of ER, AR, PR, and aryl hydrocarbon receptor occurs via degradation by the 26S proteasome (28, 51–53), these results provide compelling evidence for integration of the neddylation and ubiquitin-proteasome pathways in steroid hormone action. Because receptor levels can have a profound influence on target tissue responsiveness to hormone, NEDD8 and ubiquitin pathways, by modulating receptor protein turnover, could play important roles in determining and perhaps limiting cellular responses to steroid hormones and antihormones.

The antiestrogen ICI 182,780 is a 7α-alkylsulfinyl analog of estradiol lacking agonist activity (54). The drug is used as a second-line endocrine agent in patients who have developed tamoxifen-resistant breast cancer (38). Although the drug clearly displays complex pharmacology, rapid degradation of ERα protein has been associated with the antiproliferative effects of ICI 182,780 on breast cancer cells (38, 54). Despite its potent antitumor effects, the drug does not circumvent the development of antiestrogen resistance (55–58). Moreover, the fact that most tumors acquiring ICI 182,780 resistance do so while retaining expression of ERα and estrogen responsiveness (55, 59) suggests that administration of the antiestrogen may possibly lead to the selection of tumor cells defective in ERα down-regulation pathway(s), which in turn may confer a proliferative advantage in either the presence or absence of estrogens. Mechanism underlying persistent expression of ERα in tumors with acquired resis-

tance may thus present an important therapeutic target for future drug intervention. In this context, the loss of NEDD8 expression during malignant transformation of prostate cancer was recently reported (60). Because our results show an intact NEDD8 pathway is essential for ERα ubiquitination and degradation, we speculate that disruptions in the NEDD8 pathway may provide a mechanism by which breast cancer cells acquire ICI 182,780 resistance while retaining expression of ERα.

MATERIALS AND METHODS

Materials

The following antibodies and reagents were used in this study: anti-ER (HC20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-HA (3F10; Roche Molecular Biochemicals, Indianapolis, IN); anti-GFP (GFP01, NeoMarkers, Inc., Fremont, CA); anti-GAPDH (glyceraldehyde phosphate dehydrogenase; Chemicon International, Inc., Temecula, CA); anti-rabbit IgG and protein G-agarose beads (Oncogene Research Products, San Diego, CA); SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL); protease inhibitor cocktail set III (Calbiochem-Novabiochem Corp., San Diego, CA); Bio-Rad Laboratories, Inc. (Hercules, CA) protein assay kit; FBS and csFBS (HyClone Laboratories, Inc., Logan, UT); LipofectAMINE Plus Reagent, geneticin, and other cell culture reagents were from Life Technologies, Inc. (Rockville, MD). Estradiol, 4-OHT, MG132, and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St. Louis, MO). ICI 182,780 was purchased from Tocris Cookson Ltd. (Ellisville, MO).

Plasmid Construction

The construction of pSG5-ER(HEGO), pcDNA-Uba3, pcDNA-HA-Uba3C216S, pcDNA-HA-Ubc12, and pcDNA-HA-Ubc12C111S was described previously (25). The pcDNA-HA-ubiquitin was kindly provided by Y. Xiong (61). The pCMV (cytomegalovirus)-GFP was purchased (Promega Corp., Madison, WI).

Cell Lines

The human cervical carcinoma cell line, HeLa, and the breast cancer cell line, MCF-7 were purchased from ATCC (Manassas, VA). HeLa cells were maintained in MEM with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% FBS. MCF7 cells were maintained in MEM with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, 6 ng/ml insulin, and 10% FBS. Before experiments involving in transient transfection and hormone treatment, cells were cultured in hormone-free medium (phenol red-free MEM with 3% csFBS) for 3 d.

Transient Transfection Assays

HeLa cells were cultured in hormone-free medium for 3 d and transfected with equal amount of total plasmid DNA (adjusted by corresponding empty vectors) by using LipofectAMINE Plus Reagent according to the manufacturer's guidelines. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were cultured in hormone-free medium. All cells were also cotransfected with pCMV-GFP as internal control to correct for transfection efficiency and SDS-PAGE loading.

Stable Transfection

MCF7 cells were transfected with pcDNA-HA-Ubc12C111S or empty vector by using LipofectAMINE Plus Reagent and selected in growth medium containing 0.5 mg/ml geneticin for 3 wk. Drug-resistant colonies were chosen and expanded in growth medium containing 0.3 mg/ml geneticin. The expression of HA-Ubc12C111S in the stable cell lines (MCF7/C111S) was detected by Western blotting with anti-HA antibody. Geneticin-resistant clones from vector transfectants (MCF7/Vec) were pooled, maintained in growth medium containing 0.3 mg/ml geneticin, and used as control cells.

Preparation of Cell Extracts and Immunoblotting

Whole cell extracts were prepared by suspending cells (~2×10^6) in 0.1 ml of ice-cold lysis buffer (25 mM HEPES, pH 7.5; 0.3 M NaCl; 0.2% sodium dodecyl sulfate; 0.5% sodium deoxycholate; 0.2 mM EDTA; 0.5 mM dithiothreitol; 0.1% Triton X-100; 10 μl protease inhibitor cocktail set III). After 15 min on ice, extracts were sonicated (3 × 10 sec), insoluble material was removed by centrifugation (15 min at 12,000 × *g*), and protein concentration in the supernatant was determined using the Bio-Rad Laboratories, Inc. protein assay kit. The protein extracts were mixed with 1/4 vol of 5× electrophoresis sample buffer and boiled for 5 min at 90 C. Protein extract (50 μg per lane) was then fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with antibodies. Primary antibody was detected by horseradish peroxidase-conjugated second antibody and visualized using enhanced SuperSignal West Pico Chemiluminescent Substrate. The band density of exposed films was evaluated with ImageJ software (http://rsb.info.nih.gov/ij/).

Immunoprecipitation

For immunoprecipitation, 500 μg whole cell extract was diluted to protein concentration of 1 μg/μl using PBS containing protease inhibitor cocktail and incubated with 5 μl anti-rabbit IgG and 20 μl protein G-agarose beads for 1 h at 4 C. After centrifugation at 12,000 × *g* for 15 sec, the precleared supernatants were incubated with 5 μl anti-ER antibody overnight at 4 C, followed by another 1-h incubation with 30 μl protein G-agarose beads. The beads were then pelleted by brief centrifugation, washed three times with PBS and once with PBS containing 0.4 M NaCl, and resuspended in 30 μl SDS-PAGE loading buffer for SDS-PAGE and Western blotting.

Cell Proliferation Assays

To assess the effects of estradiol, ICI 182,780, or 4-OHT on cell proliferation, cells (1000/well) were plated in 96-well dishes in hormone-free medium for 3 d before drug exposure. For time-response analysis, cell numbers were determined by MTT assay (62) at indicated times after drug treatment; and for dose-response analysis, cell number was determined by MTT assay at d 7.

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Address all correspondence and requests for reprints to: Kenneth P. Nephew, Ph.D., Medical Sciences, Indiana

University School of Medicine, 302 Jordan Hall, 1001 East Third Street, Bloomington, Indiana 47405-4401. E-mail: knephew@indiana.edu.

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APPENDIX 2

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Inhibiting Proteasomal Proteolysis Sustains Estrogen Receptor-α Activation

MEIYUN FAN, HARIKRISHNA NAKSHATRI, AND KENNETH P. NEPHEW

Medical Sciences (M.F., K.P.N.), Indiana University School of Medicine, Bloomington, Indiana 47405; Department of Surgery (H.N.), Department of Biochemistry and Molecular Biology, Walther Oncology Center; Indiana University Cancer Center (H.N., K.P.N.); and Department of Cellular and Integrative Physiology (K.P.N.), Indiana University School of Medicine, Indianapolis, Indiana 46202

Estrogen receptor-α (ERα) is a ligand-dependent transcription factor that mediates physiological responses to 17β-estradiol (E2). Ligand binding rapidly down-regulates ERα levels through proteasomal proteolysis, but the functional impact of receptor degradation on cellular responses to E2 has not been fully established. In this study, we investigated the effect of blocking the ubiquitin-proteasome pathway on ERα-mediated transcriptional responses. In HeLa cells transfected with ERα, blocking either ubiquitination or proteasomal degradation markedly increased E2-induced expression of an ER-responsive reporter. Time course studies further demonstrated that blocking ligand-induced degradation of ERα resulted in prolonged stimulation of ER-responsive gene transcription. In breast cancer MCF7 cells containing endogenous ERα, proteasome inhibition enhanced E2-induced expression of endogenous pS2 and cathepsin D. However, inhibiting the proteasome decreased expression of progesterone receptor (PR), presumably due to the heterogeneity of the PR promoter, which contains multiple regulatory elements. In addition, in endometrial cancer Ishikawa cells overexpressing steroid receptor coactivator 1, 4-hydroxytamoxifen displayed full agonist activity and stimulated ERα-mediated transcription without inducing receptor degradation. Collectively, these results demonstrate that proteasomal degradation is not essential for ERα transcriptional activity and functions to limit E2-induced transcriptional output. The results further indicate that promoter context must be considered when evaluating the relationship between ERα transcription and proteasome inhibition. We suggest that the transcription of a gene driven predominantly by an estrogen-responsive element, such as pS2, is a more reliable indicator of ERα transcription activity than a gene like PR, which contains a complex promoter requiring cooperation between ERα and other transcription factors. (*Molecular Endocrinology* 18: 2603–2615, 2004)

THE ACTIONS OF estrogens are mediated primarily through estrogen receptors (ERα and ERβ) (1), ligand-dependent transcription factors that interact directly with estrogen response elements (EREs) in the promoters of target genes (1). Cellular levels of ERα (2), along with a large number of receptor coregulator complexes (3), play key roles in controlling appropriate physiological responses in estrogen target tissues, such as breast and uterus. Levels of ERα mRNA and protein are regulated primarily by its cognate ligand, 17β-estradiol (E2) (4–6). E2 binding results in rapid turnover of ERα protein through the ubiquitin (Ub)-proteasome pathway (7–11), which has been implicated in both the overall control of gene transcription (12–16) and transactivation function of ERα and other nuclear receptors (7, 17–24).

The Ub-proteasome system consists of the 26S proteasome, a complex composed of a 20S catalytic core for protein proteolysis and two ATPase-containing 19S regulatory particles that recognize polyubiquitin-tagged substrates (25). Like many other transcription factors, stimulation of ERα transcriptional activation appears to be associated with receptor ubiquitination and proteasomal degradation (11, 26). Several proteins possessing Ub ligase activity (*e.g.* E6AP, p300, BRCA1, and MDM2), as well as SUG1, a component of the 19S proteasome, have been shown to associate with ERα and modulate receptor signaling (27–34). These observations suggest that proteasome-mediated receptor degradation is important for ER function.

Recent studies have demonstrated that inhibiting proteasomal degradation increases transcriptional activity of many, but not all, nuclear receptors, indicating a receptor-specific effect of proteasome inhibition (17–24). Blocking ERα turnover by a proteasome-

Abbreviations: CAT, Chloramphenicol acetyltransferase; DMSO, dimethylsulfoxide; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response elements; GR, glucocorticoid receptor; hnRNA, heterogeneous nuclear RNA; Luc, firefly luciferase; 4-OHT, 4-hydroxytamoxifen; p-Pol II, phosphorylated RNA pol II; PR, progesterone receptor; Q-PCR, real-time quantitative reverse transcription-PCR; RSV, Rous sarcoma virus; SRC, steroid receptor coactivator; SV40, simian virus 40; Ub, ubiquitin; Ubc, ubiquitin-conjugation enzyme; Vit, vitellogenin.

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specific inhibitor, MG132, results in decreased expression of an ERα-responsive luciferase reporter, implicating that proteasomal degradation of ERα is required for its transactivation function (7, 35). However, MG132, and other proteasome inhibitors, have recently been shown to deleteriously affect production of a functional firefly luciferase enzyme (36), complicating the assessment of studies utilizing only ERα-responsive reporters expressing luciferase, in combination with 20S proteasome inhibitors. In addition, several studies have recently suggested that receptor degradation may not be required for ERα-mediated transcription. Frasor *et al.* (11, 37) reported that the partial agonist/antagonist 4-hydroxytamoxifen (4-OHT), which protects ERα from proteasomal degradation, stimulates ER-mediated transcription of a group of genes in MCF7 cells (38). Dissociation of ERα activation from degradation has also been reported in pituitary tumor cells (39, 40).

In the present study, we investigated the role of the Ub-proteasome pathway in ERα-mediated transcriptional responses. Genetic and pharmacological approaches were used to disrupt ERα ubiquitination, proteasome-mediated proteolysis, and thus ERα degradation, including the 20S proteasome inhibitor MG132, a dominant-negative mutant of the NEDD8 conjugation enzyme (Ubc12C111S) (41, 42), a Ub mutant with all of its lysines mutated to arginine (UbK0) (43), and the partial agonist/antagonist 4-OHT. To determine the effect of blocking ERα degradation on E2-induced transcriptional responses, ER-responsive reporter assays and expression of endogenous ER-target genes were used. The results demonstrate that proteasomal degradation is not essential for transcriptional activity of ERα and indicate that the Ub-proteasome system functions to limit E2-induced transcriptional output.

RESULTS

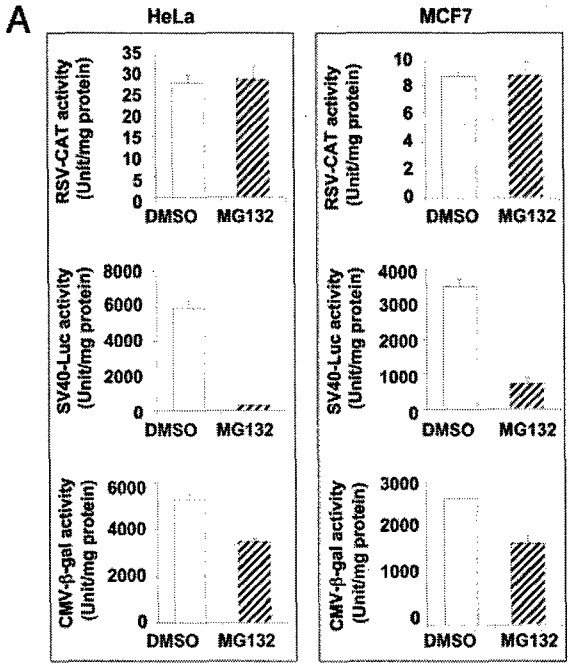
Inhibiting the Proteasome Increases ERα Transcriptional Output

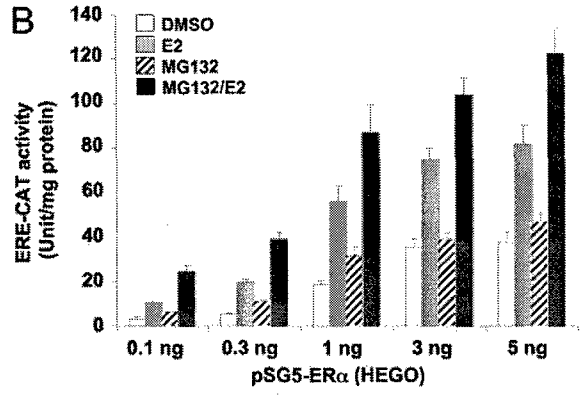
The enzymatic activity of chloramphenicol acetyltransferase (CAT), luciferase (Luc) or β-galactosidase (Gal) reporter proteins is commonly used for assessing transcriptional activity of nuclear receptors in the presence of proteasome inhibitors. Recent studies with breast cancer T47D cells revealed that proteasome inhibitors (MG132, lactacystin, and proteasome inhibitor I) interfere with the production of luciferase and galactosidase proteins by a posttranscriptional mechanism, whereas the enzymatic activity of CAT remains unaffected (36). To verify these observations in our experimental systems, we examined the effect of MG132 on expression of these reporter enzymes from constitutively active constructs in cervical carcinoma HeLa and breast cancer MCF-7 cells. Cells were transfected with Rous sarcoma virus (RSV)-CAT, simian virus 40 (SV40)-Luc, or cytomegalovirus (pCMV)-β-gal and then treated with vehicle [dimethylsulfoxide (DMSO)] or MG132 (1 μM) for 24 h. Reporter enzyme activity was determined using standard assays for luciferase, CAT, and galactosidase. Treatment of HeLa cells with MG132 had no effect on CAT activity but decreased luciferase and galactosidase activity by 80% and 30%, respectively (Fig. 1A, *left panel*). Essentially similar results were obtained using MCF7 cells (Fig. 1A, *right panel*). These results agree with a previous report demonstrating that proteasome inhibitors have deleterious effects on the enzymatic activities of luciferase and galactosidase reporter proteins (36).

Previously, we and others showed that E2 induces ERα degradation in transiently transfected HeLa cells and MG132 abolishes such degradation (8, 9, 42). Based on the above results, we further investigated the relationship between ERα turnover and E2-induced transcriptional response using an E2-responsive CAT reporter. HeLa cells were transiently transfected with ERE-vitellogenin (Vit)-CAT and different doses of ERα-expressing construct (0.1–5 ng pSG5-ERα/10^5 cells). Cells were treated with vehicle (DMSO) or MG132 (1 μM) for 1 h followed by E2 (10 nM). CAT activity was measured 24 h after E2 treatment. Basal CAT activity increased, proportional to the amount of pSG5-ERα (Fig. 1B; *open bars*). As expected, E2 markedly induced CAT activity (Fig. 1B; *gray bars*); however, treatment with MG132 plus E2 resulted in greater CAT activity, compared with E2 alone (Fig. 1B; *black vs. gray bars*). Cells treated with MG132 alone exhibited slightly higher CAT activity than the DMSO control (Fig. 1B, *hatched bars*). A synergistic effect of MG132 plus E2 was observed in cells transfected with lower levels of ERα (0.1–0.3 ng pSG5-ERα/10^5 cells). For example, the combined treatment of MG132 and E2 increased ERE-CAT activity by about 7.4-fold in cells transfected with 0.1 ng pSG5-ERα/10^5 cells, whereas MG132 or E2 alone increased ERE-CAT activity by 1.82- or 3.10-fold, respectively (*table* in Fig. 2B). Immunoblot analysis showed that pretreatment with MG132 effectively blocked E2-induced ERα down-regulation in HeLa cells (Fig. 1C). Taken together, these observations demonstrate that ERα retains the capacity to activate transcription in the absence of proteasomal degradation, and blocking ERα turnover increases E2-induced transcriptional output. The results further suggest that, in cells containing low levels of ERα, proteasome-mediated receptor degradation plays a role in limiting E2-induced transcriptional responsiveness.

Effect of Inhibiting the Proteasome on E2 Sensitivity

Based on the observation that preventing receptor protein turnover increases ERα-mediated transcription, we examined the effect of inhibiting the proteasome on hormone sensitivity. HeLa cells were transfected with ERE-Vit-CAT and pSG5-ERα,





The fold increases of ERE-CAT (relative to DMSO)

	pSG5-ERα				
	0.1 ng	0.3 ng	1 ng	3 ng	5 ng
DMSO	1.00	1.00	1.00	1.00	1.00
E2	3.10	3.70	3.00	2.09	2.17
MG132	1.82	2.01	1.68	1.08	1.22
MG132/E2	7.40	7.31	4.63	2.91	3.23

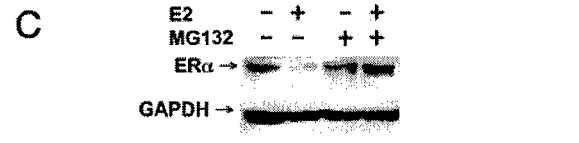


Fig. 1. Proteasome Inhibition Enhances E2-Induced CAT Reporter Gene Expression in HeLa Cells Transfected with ERα

A, Effect of proteasome inhibition by MG132 on expression of reporter enzymes from constitutively active promoters. HeLa cells (*left panel*) were plated on 12-well dishes at a density of 1×10^5 cells per well and cultured in hormone-free medium for 3 d. The cells were transfected with 100 ng RSV-CAT, 100 ng SV40-Luc, or 5 ng pCMV-β-gal using LipofectAMINE Plus Reagent. The DNA/LipofectAMINE mixture was removed 5 h later and cells were placed in hormone free medium containing either 0.1% vehicle (DMSO) or 1 μM MG132 for 24 h. Similarly, MCF7 cells (*right panel*) were treated with DMSO or MG132 for 1 h, and then treated with various doses of E2 (1×10^{-15} to 1×10^{-8} M). CAT activity was determined 24 h after the addition of ligand. In cells transfected with 0.3 ng (Fig. 2A) or 1 ng pSG5-ERα (Fig. 2B), a hyperbolic dose response to E2 was observed; the lowest dose of hormone that induced CAT activity was 1×10^{-11} M E2. Increasing ERα expression (0.3 ng *vs.* 1 ng pSG5-ERα) and pretreatment with MG132 augmented maximal CAT induction by E2, but no effect on E2 sensitivity was observed. The minimal dose of E2 required to induce CAT was 1×10^{-11} M under all experiment conditions, and the EC_{50} was not different (Fig. 2). These results demonstrate that blocking ERα degradation increases the magnitude of E2-induced gene transcription but has no effect on hormone sensitivity.

Inhibiting the Proteasome Extends the Duration of E2-Induced Gene Transcription

The results of the above experiments suggest that inhibiting the proteasome may extend the half-life of ligand-activated ERα and thus increase receptor transcriptional output. To test the possibility that MG132 treatment would subsequently extend the duration of an E2-induced transcriptional response, we performed a time course analysis using luciferase as a reporter protein. The half-life of CAT in mammalian cells is about 50 h (44);

plated at a density of 1.2×10^5 cells per well, transfected with 250 ng RSV-CAT, 250 ng SV40-Luc, or 10 ng pCMV-β-gal and then treated with DMSO or MG132 for 24 h. Reporter enzyme activities were normalized against total cellular protein and expressed as the mean ± SD from three independent experiments, each in triplicate. B, Effect of MG132 on ERα-mediated CAT expression. HeLa cells were plated in 12-well dishes at a density of 1×10^5 cells per well and cultured in hormone-free medium for 2 d. The cells were transfected with 100 ng ERE-Vit-CAT and the indicated amount of pSG5-ERα using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later and cells were placed in hormone-free medium for 24 h. Transfected cells were treated with DMSO or MG132 (1 μM) for 1 h and then treated with 10 nM E2 for 24 h. CAT activity was determined using the colorimetric CAT ELISA kit and normalized against total cellular protein. CAT activity is expressed as the mean ± SD of three independent experiments, each performed in triplicate. Fold increases in ERE-CAT in the presence of E2 ± MG132 are presented in the *table*. C, Effect of MG132 on E2-induced down-regulation of ERα. HeLa cells were plated in 60-mm dishes at a density of 3×10^5 cells per dish and cultured in hormone-free medium for 2 d. Cells were transfected with 100 ng pSG5-ERα using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later, and cells were placed in hormone-free medium for 24 h. The transfected cells were treated with DMSO or MG132 (1 μM) for 1 h and then treated with 10 nM E2 for 8 h. Whole-cell lysates were prepared and subjected to immunoblotting analysis using an anti-ERα antibody (Chemicon, Temecula, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

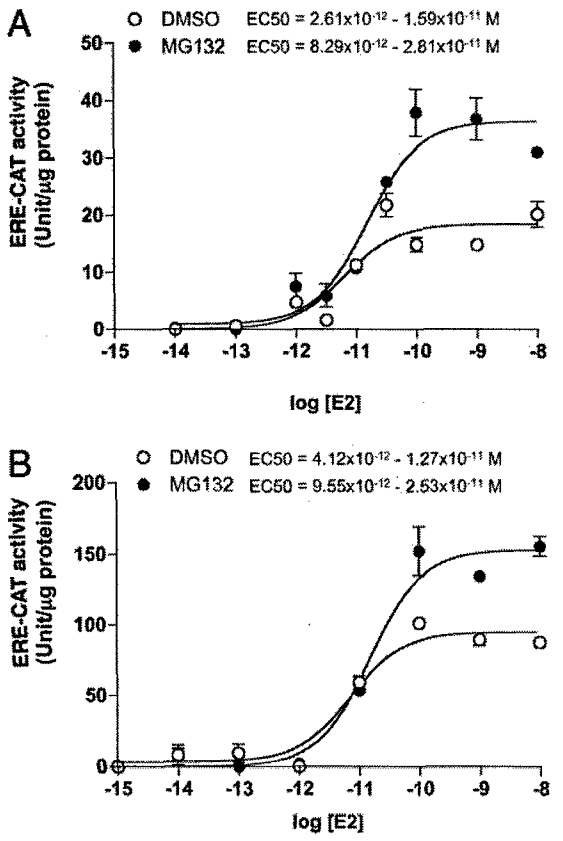


Fig. 2. Effect of MG132 on E2 Dose-Dependent Induction of Reporter Gene Expression in HeLa Cells

HeLa cells were plated in 12-well dishes at a density of 1×10^5 cells per well and cultured in hormone-free medium for 2 d. The cells were transfected with 100 ng ERE-Vit-CAT and 0.3 ng (A) or 1 ng (B) of pSG5-ERα using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later and cells were placed in hormone-free medium for 24 h. The transfected cells were treated with DMSO or MG132 (1 μM) for 1 h and then treated with the indicated concentration of E2 for 24 h. CAT activities were normalized against total cellular protein and expressed as mean ± SD of three independent experiments, each performed in triplicate. EC_{50} range was calculated with a 95% confidence.

in contrast, luciferase has an intracellular half-life of about 3 h (44), making it well suited for performing a dynamic analysis of promoter activation. Thus, we used HeLa cells transfected with ERα and ERE-pS2-Luc to study the effect of proteasome inhibition on E2-induced transcription in a time-dependent manner. In transfected HeLa cells, E2 induced a transient induction of luciferase activity, maximal at 6 h (Fig. 3A, *solid circles*). Pretreatment with MG132 decreased E2-induced luciferase expression at the early time points (1.5–6 h), but markedly increased E2-induced luciferase expression from 9–20 h (Fig. 3A, *solid triangles*).

As mentioned above, MG132 can inhibit luciferase production. To determine the effect of MG132 on luciferase synthesis in general, we transfected HeLa cells with a constitutively active luciferase construct (SV40-Luc). In contrast to what we observed using ERE-pS2-Luc, MG132 consistently decreased the expression of SV40-Luc during the 20-h period (Fig. 4B), excluding the possibility that MG132 enhances ERE-luc activity by stabilizing luciferase protein. To subtract the general inhibitory effect of MG132 on luciferase synthesis, at each time point shown in Fig. 3C, ERα-mediated luciferase expression in the presence of MG132 was normalized to luciferase activity from the SV40-Luc construct [normalized ERE-Luc activity in the presence of MG132 = ERE-Luc activity in the presence of MG132 × (SV40-Luc activity/SV40-Luc activity in the presence of MG132)]. The adjusted results clearly demonstrate that blocking receptor degradation with MG132 increases both the magnitude and duration of E2-induced gene transcription, suggesting that the duration of gene transcription induced by E2 is limited by ERα degradation through the 26S proteasome.

Inhibiting ERα Ubiquitination Prolongs E2-Induced Gene Transcription

In a previous study, we used a dominant-negative mutant of the NEDD8 conjugation enzyme, Ubc12C111S, to inhibit ERα ubiquitination and degradation (42). Here we used Ubc12C111S as a means to investigate the role of ERα turnover in ERα transactivation function and to corroborate our observations using MG132. The impact of Ubc12C111S on the time-dependent induction of a reporter gene by ERα was investigated. HeLa cells were transfected with pSG5-ERα and ERE-pS2-Luc, along with a control vector (pcDNA) or a construct expressing the mutant Ubc12 (pcDNA-Ubc12C111S). In cells transfected with pcDNA, E2 transiently induced luciferase expression, and maximal induction was observed at 5 h (Fig. 3D, *solid circles*). However, in cells transfected with pcDNA-Ubc12C111S, a delay in peak expression of E2-induced luciferase activity was observed (9 h; Fig. 3D, *solid triangles*), and luciferase expression remained elevated, even 20 h after E2 treatment. No effect of Ubc12C111S on maximal E2-induced luciferase activity was observed (Fig. 3D, *solid circles vs. solid triangles*). To confirm that the observed effect of Ubc12C111S on ERα-mediated luciferase expression was specific, luciferase activity in cells cotransfected with SV40-Luc and Ubc12C111S was assessed over time. No effect of Ubc12C111S on SV40-Luc expression was seen at 6 and 12 h after transfection; a slight increase in luciferase expression was observed at 20 h (1.3-fold; Fig. 3E). Overall, these results demonstrate that inhibiting ERα ubiquitination prolongs ERα-mediated transcription, supporting the hypothesis that proteasome-mediated degradation of ERα serves as a means to limit the duration of E2 signaling.

Blocking Polyubiquitination Sustains E2-Induced Gene Expression

To determine the effect of blocking polyubiquitination on ERα-mediated transcription, we used a Ub mutant,

UbK0, which has all of its lysines replaced by arginine. This mutant competes with endogenous ubiquitin and terminates ubiquitin chains, resulting in the accumulation of short ubiquitin conjugates that cannot be degraded efficiently by the proteasome (43). First, we examined the effect of overexpressing UbK0 on E2-

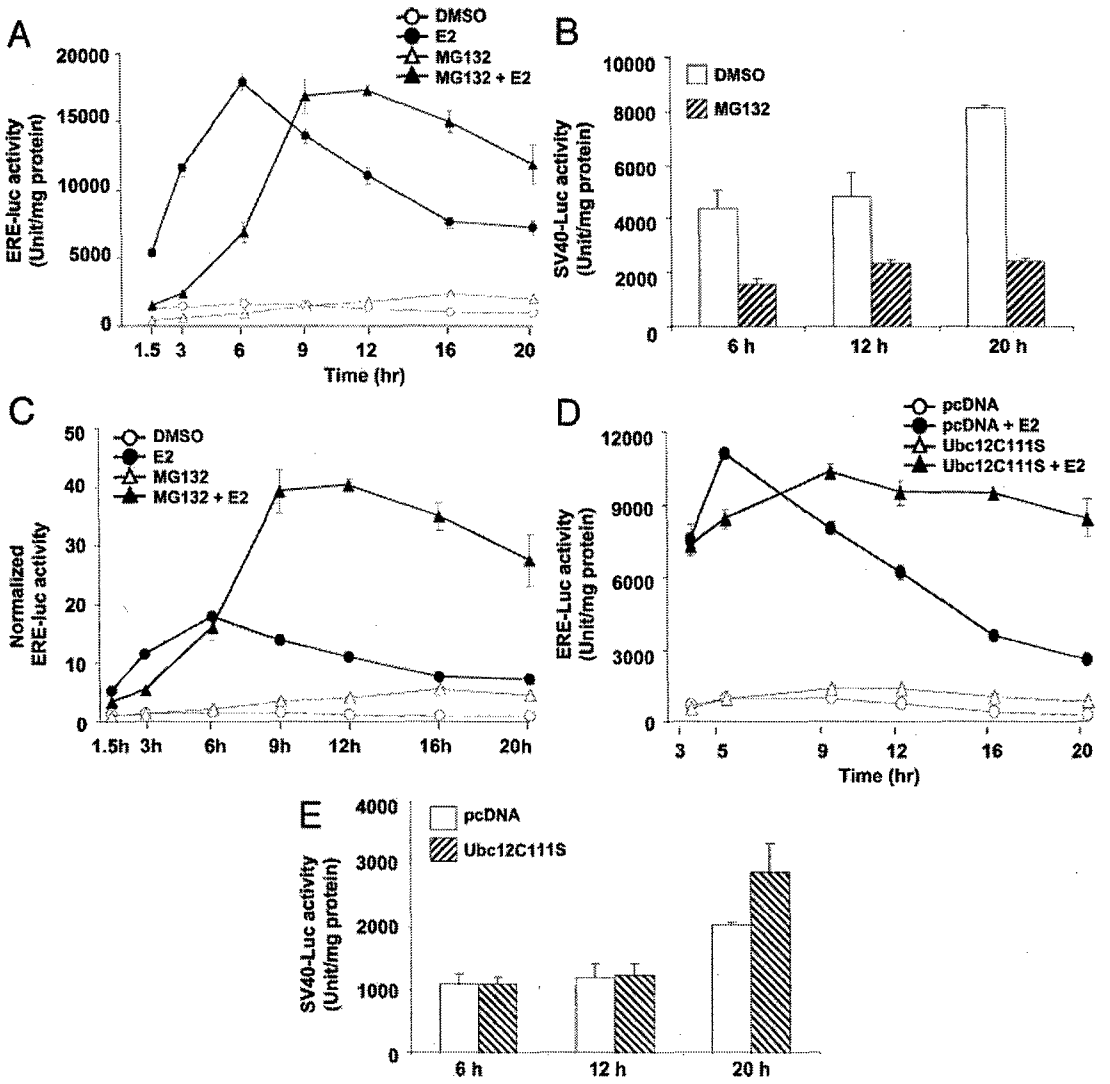


Fig. 3. Effect of Blocking ERα Turnover on Time-Dependent Induction of Reporter Gene Expression by E2 in HeLa Cells

A, Effect of MG132 on E2-induced expression of reporter gene. HeLa cells were plated in 12-well dishes at a density of 1×10^5 cells per well and cultured in hormone-free medium for 2 d. The cells were transfected with 250 ng ERE-pS2-Luc and 1 ng of pSG5-ERα using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later, and cells were placed in hormone-free medium for 24 h. The transfected cells were treated with DMSO or MG132 (5 μM) for 1 h and then treated with 10 nM E2 for the indicated time period. Luciferase activity was determined using the Luciferase Assay System, normalized against total cellular protein. B, Effect of MG132 on SV40-Luc expression. HeLa cells were transfected with 100 ng SV40-Luc. The DNA/LipofectAMINE mixture was removed 5 h later and cells were placed in hormone-free medium containing either 0.1% vehicle (DMSO) or MG132 (5 μM) for the indicated time period. Luciferase activity was determined and normalized against total cellular protein. C, Normalized ERE-Luc activities. ERα-mediated luciferase activity in the presence of MG132 was normalized to luciferase activity from the SV40-Luc construct [Normalized ERE-Luc activity in the presence of MG132 = ERE-Luc activity in the presence of MG132 × (SV40-Luc activity/SV40-Luc activity in the presence of MG132)]. D, Effect of overexpressing Ubc12C111S on E2-induced reporter gene expression. HeLa cells were transfected with 250 ng ERE-pS2-Luc, 1 ng pSG5-ERα, along with 100 ng pcDNA or pcDNA-Ubc12C111S, and treated with 10 nM E2 for the indicated period of time. Luc activities were normalized against total cellular protein. E, Effect of overexpressing Ubc12C111S on SV40-Luc expression. HeLa cells were transfected with 100 ng SV40-Luc, along with 100 ng pcDNA-Ubc12C111S or control vector pcDNA. The DNA/LipofectAMINE mixture was removed 5 h later, and cells were placed in hormone-free medium for the indicated time period. Luc activities were normalized against total cellular protein. For all assays, Luc activities are expressed as mean ± SD from three independent experiments, each performed in triplicate.

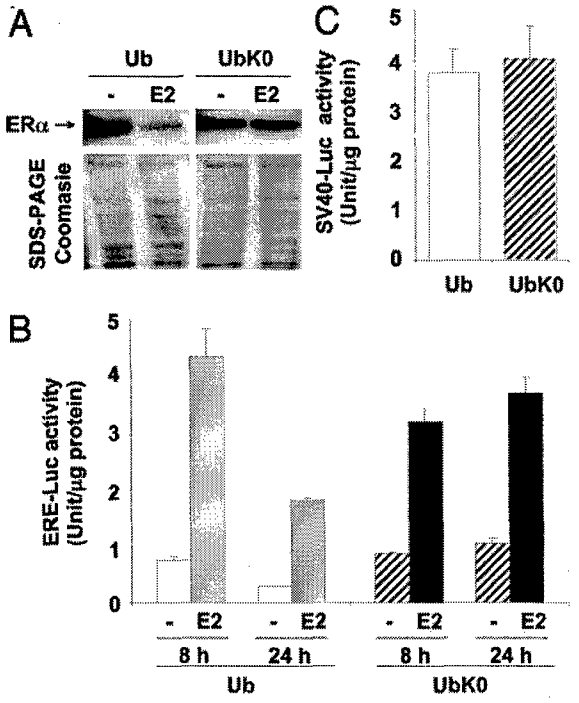


Fig. 4. Ub Mutant Blocks ER Degradation and Sustained E2-Induced Gene Expression

A, Overexpression of UbK0 blocks E2-induced ERα degradation. HeLa cells were plated in 60-mm dishes at a density of 3×10^5 cells per dish and cultured in hormone-free medium for 2 d. The cells were transfected with 150 ng pSG5-ERα, along with 150 ng pcDNA-Ub or pCS2-UbK0, using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later, and cells were placed in hormone-free medium for 24 h before treatment with DMSO or 10 nM E2 for 8 h. Whole-cell lysates were prepared and subjected to immunoblotting analysis using an anti-ERα antibody. The Coomasie-stained SDS-PAGE gels show that equal amounts of cell lysates were loaded. B, Effect of UbK0 on ERα-mediated luciferase expression. HeLa cells stably transfected with ERα were plated in 12-well dishes at a density of 1×10^5 cells per well and cultured in hormone-free medium for 2 d. The cells were transfected with 250 ng ERE-pS2-Luc, along with 100 ng pcDNA-Ub or pCS2-UbK0 as indicated, using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later, and cells were placed in hormone-free medium for 24 h before treatment with DMSO or 10 nM E2 for the indicated time period. C, Effect of UbK0 on luciferase expression from SV40-Luc. HeLa cells stably transfected with ERα were transfected with 100 ng SV40-Luc, along with 100 ng pcDNA-Ub or pCS2-UbK0. Five hours later, the DNA/LipofectAMINE mixture was removed, and cells were placed in hormone-free medium for the indicated time period. Luciferase activity was normalized against total cellular protein and expressed as the mean ± SD from three independent experiments, each performed in triplicate.

induced ERα degradation. In HeLa cells cotransfected with wild-type Ub and ERα, the level of receptor protein decreased markedly after E2 treatment (Fig. 4A), accompanied by transient E2-induced expression of an ER-responsive luciferase reporter gene (Fig. 4B, 8 h *vs.* 24 h). In contrast, cells transfected with UbK0 showed sustained E2-induced luciferase expression (Fig. 4B), and no decrease in ERα protein levels was observed (Fig. 4A). Furthermore, the effect of UbK0 on ERα-induced luciferase was specific, as UbK0 showed no effect on expression of the SV40-Luc construct (Fig. 4C). These results demonstrate that blocking polyubiquitination of ERα stabilizes the receptor, resulting in the prolonged expression of an ERα-responsive gene.

Proteasome Inhibition Enhances ERα-Mediated Transcription in MCF7 Breast Cancer Cells

To further investigate the role of ERα degradation in receptor transactivation ability under physiologically relevant conditions, we examined the effect of inhibiting the proteasome in MCF7 breast cancer cells, which endogenously express ERα. First, we examined the effect of MG132 on ERE-Vit-CAT expression in MCF7 cells. MCF7 cells were transiently transfected with ERE-Vit-CAT and then treated with DMSO or MG132 (1 μM) for 1 h before E2 (10 nM) treatment. CAT activity was determined 24 h after E2 treatment. A 17.8 ± 1.7 fold increase in CAT expression was seen in MCF7 cells treated with E2, compared with the control; treatment with MG132 further increased E2-induced CAT activity to 25.6 ± 2.5 fold. Therefore, inhibiting the proteasome enhanced ERα transcriptional activity in MCF7 cells, indicating that ERα degradation plays a key role in limiting E2-induced transcriptional responses in breast cancer cells.

To determine the effect of proteasome inhibition on transcription of ERα-target genes in breast cancer cells, we pretreated MCF7 cells with MG132 and examined E2-induced pS2 gene expression. ERα regulates pS2 transcription through an imperfect palindromic ERE at position −405 to −393 of its promoter region (45); pS2 expression is considered a reliable indicator of ERα transcriptional activity (46). Time-dependent effects of MG132 on heterogeneous nuclear pS2 RNA (pS2 hnRNA) levels, which reflect the rates of pS2 gene transcription (47–50), were examined. Primers amplifying the conjoining sequence between the first intron and second exon of the pS2 gene were used, and expression of pS2 hnRNA was assessed by real-time quantitative RT-PCR (Q-PCR). After administration of E2, levels of pS2 hnRNA increased by 3 h, peaked at 12 h, and then declined by 70% during the next 8 h (Fig. 5A, *gray bars*). However, at all time points examined, E2-induced expression of pS2 hnRNA was markedly enhanced by pretreatment with MG132 (Fig. 5A, *black vs. gray bars*), and pS2 hnRNA levels declined only by 15% from 12–20 h after the combined treatment (Fig. 5A, *black bars*). MG132 alone showed no effect on basal pS2 hnRNA expression (Fig. 5A, *hatched bars*). In agreement with what we observed with pS2 hnRNA, the combined treatment of MG132 plus E2 resulted in greater expression of pS2 mRNA after 6 h, compared with E2 treatment

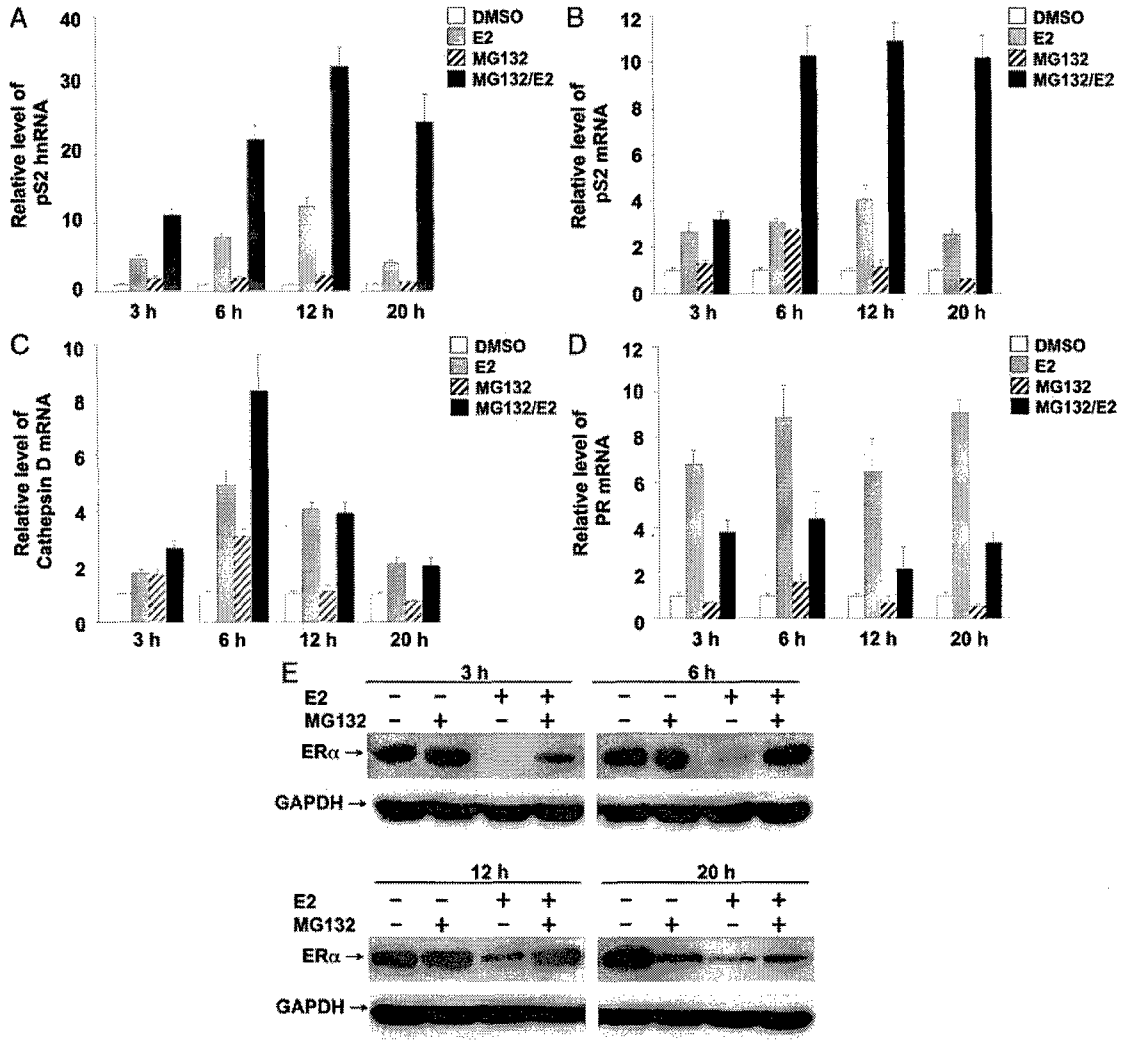


Fig. 5. Effects of MG132 on ERα-Mediated Transcription of Endogenous Target Genes in MCF7 Cells

MCF7 cells were plated at a density of 3×10^6 per 10-cm dish and allowed to grow in hormone-free medium for 3 d. The cells were pretreated with MG132 (5 μM) for 1 h and then treated with 10 nM E2 for the indicated time periods. Total RNA was prepared and subjected to Q-PCR analysis to determine the expression levels of pS2 hnRNA (A), pS2 mRNA (B), cathepsin D mRNA (C), and PR mRNA (D). For all Q-PCR assays, the relative levels of mRNA were normalized with β-actin mRNA and standardized such that values obtained in cells treated with vehicle (DMSO) only were set to 1. The results were expressed as mean ± SD from two independent experiments, each in duplicate. To determine the effect of MG132 on E2-induced ER degradation, MCF7 cells were treated as in panel A and subjected to whole-cell lysate preparation and immunoblotting with an anti-ER antibody (E). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

alone (Fig. 5B, *black vs. gray bars*); pS2 mRNA levels remained markedly elevated up to 20 h, the last time point examined (Fig. 5B, *black bars*). The coordinate increase in E2-induced expression of both pS2 hnRNA and pS2 mRNA by MG132 excludes the possibility that MG132 inhibits the hnRNA splicing process or stabilizes pS2 mRNA. Therefore, it seems reasonable to conclude that blocking the proteasome with MG132 enhances E2-induced pS2 transcription initiation. Together, these results demonstrate that inhibiting the proteasome increases both the magnitude and duration of E2-induced expression of the endogenous pS2 gene in breast cancer cells.

We also examined the effect of MG132 on mRNA expression of cathepsin D and progesterone receptor (PR), two well-known E2-regulated genes, in MCF7 cells. As shown in Fig. 5C, a transient increase in cathepsin D mRNA expression was observed after treatment with E2. Pretreatment with MG132 enhanced both basal and E2-induced cathepsin D expression at 3 and 6 h (Fig. 5C, *black vs. gray bars*); however, at 12 and 24 h, the effect of MG132 was no longer apparent. Treatment of MCF7 cells with E2 increased PR mRNA levels 7-fold by 3 h, and PR mRNA levels remained elevated throughout the experiment period (Fig. 5D, *gray bars*). MG132 pretreatment

decreased E2-induced expression of PR mRNA by more than 50% at all time points examined (Fig. 5D, *black vs. gray bars*), which agrees with a recent report that MG132 inhibits ERα-induced increase in PR protein levels (7). The differential effects of MG132 on these ERα-target genes demonstrate that promoter context must be considered when evaluating MG132 regulation of ERα-mediated transcription. Immunoblotting analysis showed that pretreatment with MG132 efficiently blocked E2-induced ERα down-regulation in MCF7 cells (Fig. 5E).

4-OHT Stimulates ERα-Mediated Transcription without Inducing ERα Degradation

The antiestrogen 4-OHT has been shown to up-regulate ERα levels by blocking ERα degradation (37), and previous studies have shown that 4-OHT functions as an ERα agonist in Ishikawa endometrial cancer cells (51, 52). To further examine the relationship between receptor stability and ERα-mediated transcription, we stably transfected ERα-negative Ishikawa cells with ERα. The ERα(+) Ishikawa cells were then transfected with a luciferase reporter construct containing the human C3 promoter (C3T1-Luc) and then treated with either E2 (10 nM) or 4-OHT (1 μM) for 16 h. After E2 administration, a 2-fold increase in luciferase activity was observed (Fig. 6A), accompanied by a marked decrease in ERα protein level (Fig. 6B). Treatment with 4-OHT also stimulated expression of luciferase (80% of E2-stimulated luciferase expression) (Fig. 6A), but the antiestrogen did not down-regulate ERα (Fig. 6B). Thus, these results demonstrate that the partial agonist activity of 4-OHT and ERα degradation are not coupled in endometrial cancer cells. It has been reported that steroid receptor coactivator 1 (SRC-1), by stimulating transcription activity of 4-OHT liganded ERα (53), can convert 4-OHT to a full agonist. We reasoned that if receptor degradation is essential for ERα to initiate transcription, SRC1 should enhance 4-OHT-stimulated ERα transactivation activity and, in parallel, induce proteasomal degradation of 4-OHT-liganded ERα. To test this reasoning, the ERα(+)Ishikawa cells

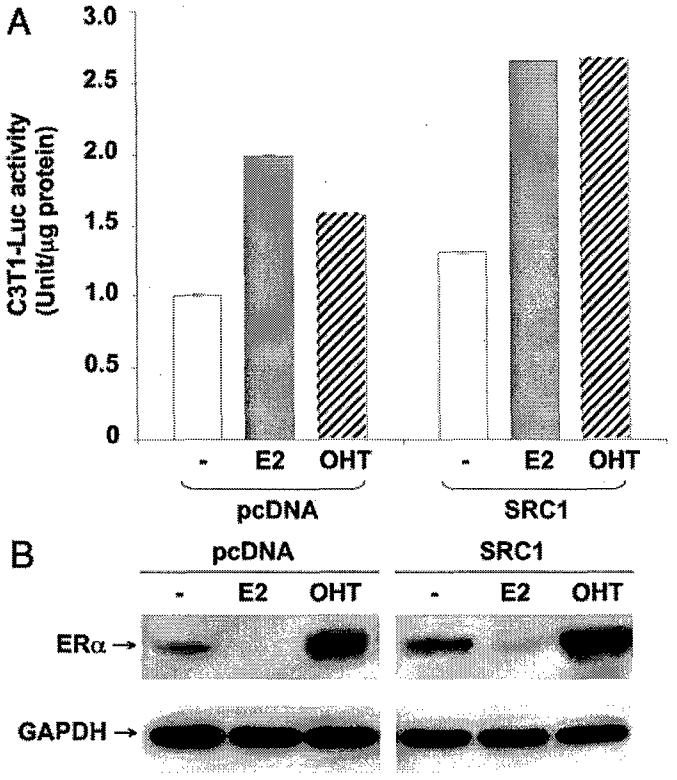


Fig. 6. Uncoupling of 4-OHT-Induced ERα Activation and ERα Degradation

A, 4-OHT stimulates ERα-mediated gene expression in Ishikawa cells. Ishikawa cells stably transfected with ERα were plated in 12-well dishes at a density of 1×10^5 cells per well and cultured in hormone-free medium for 2 d. The cells were transfected with 250 ng C3T1-Luc, along with 100 ng pcDNA or pcDNA-SRC1, using LipofectAMINE Plus reagent. The DNA/LipofectAMINE mixture was removed 5 h later, and cells were placed in hormone-free medium for 24 h before treatment with 10 nM E2 or 1 μM 4-OHT for 16 h. Luciferase activity was normalized against total cellular protein and expressed as mean ± SD from three independent experiments, each performed in triplicate. B, Effect of 4-OHT on ERα protein level. Ishikawa cells stably transfected with ERα were plated in 60-mm dishes at a density of 3×10^5 cells per dish and cultured in hormone-free medium for 3 d before treatment with 10 nM E2 or 1 μM 4-OHT for 16 h. Whole-cell lysates were prepared and subjected to immunoblotting analysis using an anti-ER antibody. GAPDH was used as a loading control.

were cotransfected with a construct expressing SRC1 and C3T1-Luc and then treated with either E2 (10 nM) or 4-OHT (1 μM) for 16 h. As expected, overexpressing SRC1 resulted in similar 4-OHT- and E2-stimulated ERα activity (Fig. 6A); however, 4-OHT did not induce receptor down-regulation (Fig. 6B). Thus, under these experimental conditions, 4-OHT, even when behaving as a full agonist in the presence of an increased level of SRC-1, did not induce ERα degradation. Taken together, these results demonstrate that ERα-mediated gene transactivation can be uncoupled from receptor degradation.

DISCUSSION

Like other rapidly turned over transcription factors, engagement of ERα in transactivation is coupled to ERα degradation by the Ub-proteasome pathway (7–11, 35). However, the functional impact of ERα degradation on cellular responses to E2 has not been well established. In this study, we analyzed the effect of blocking ERα degradation on E2-induced transcriptional output. We demonstrate that blocking ERα turnover prolongs the ability of ERα to transactivate target genes and increases the output of E2-induced gene transcription. We also show that 4-OHT can act as a full agonist in Ishikawa cells overexpressing SRC-1 to stimulate ERα transcriptional activity, without inducing receptor degradation. Furthermore, proteasome inhibition by MG132 increases ERα-mediated reporter gene expression, as well as expression of endogenous ERα-target genes (pS2 and cathepsin D), in MCF7 breast cancer cells. These data demonstrate that proteasomal degradation is not essential for ERα transcriptional activity; ERα remains functional after escaping ubiquitination and proteasomal proteolysis. An important implication of this study is that the E2-induced transcriptional response is limited by receptor degradation through the Ub-proteasome system, and defects in proteasome-mediated degradation of ERα could lead to an enhanced cellular response to E2.

In this study, several approaches targeting different steps in ubiquitination/proteasome proteolysis were used to block ERα degradation. MG132 was used to inhibit ERα proteolysis by specifically blocking activity of the 20S proteasome. A dominant-negative mutant (Ubc12C111S) of the NEDD8 conjugation enzyme was used to block ERα ubiquitination by inhibiting Ub ligase activity (41, 42). A Ub mutant with all of its lysines mutated to arginine (UbK0) was used to block ERα polyubiquitination by terminating polyubiquitin chains (43). One concern regarding the use of these approaches is a lack of specificity, such that the observed effect on enhanced E2-induced transcriptional output could be due to stabilization of multiple regulatory proteins, in addition to ERα. However, several observations suggest that this is not the case. MG132, Ubc12C111S, and UbK0 substantially enhance E2-induced, but not basal, expression of ERE reporter genes or the endogenous pS2 gene, suggesting that the effect of these inhibitors on ERα target gene expression is hormone dependent and thus receptor dependent. Furthermore, a time-dependent effect on E2-induced gene transcription was observed, which agrees with the ability of these inhibitors to block ligand-induced ERα degradation. Finally, no time-dependent effect on SV40-Luc expression was observed, in contrast to ERE-Luc, suggesting that these inhibitors do not broadly affect gene transcription in a time-dependent manner. Therefore, we conclude that MG132, Ubc12C111S, and UbK0 enhance E2-induced gene transcription primarily by extending the lifetime of functional ERα.

Consistent with our ERα findings, proteasome inhibition has been shown to enhance the transcriptional response mediated by other nuclear receptors, including the glucocorticoid receptor (GR) (17, 24), aryl hydrocarbon receptor (18), peroxisome proliferator-activated receptor α (19), retinoid receptors (20), and the vitamin D_3 receptor (21). However, it has also been reported that MG132 decreases transcriptional activity of PR and androgen receptor (22, 23), indicating that the effect of proteasome inhibition on transcriptional activity could be receptor specific. This is presumably due to the involvement of mechanisms other than modulation of receptor levels; for example, MG132 inhibited androgen receptor activity by eliminating androgen-induced nuclear translocation and coactivator recruitment (22, 23).

In MCF7 cells, we observed differential effects of MG132 on E2-induced transcription of endogenous pS2, cathepsin D, and PR gene, suggesting that proteasome inhibition can have promoter-specific effects on gene transcription. Although the reason for this is not clear, these observations raise the intriguing possibility of a differential requirement of ERα turnover in gene transcription, such that ERα degradation is required for PR transcription, but not for pS2 and cathepsin D. However, another attractive possibility is that multiple regulatory elements, other than an ERE, could be differentially regulated by proteasome inhibition; the different structures of the PR, pS2, and cathepsin D promoters may favor this possibility. For endogenous genes, the effect of estrogen is usually mediated through cross-talk between the ERE and nearby regulatory elements, and there appears to be an inverse correlation between the influence of nearby elements and the strength of the ERE (54). The ERE sequence in pS2 promoter deviates from the consensus palindromic ERE by 1 bp and, when isolated from surrounding sequences, is able to mediate estrogen responsiveness (45); however, for the cathepsin D promoter, although the ERE-like sequence deviates from the consensus ERE by only 2 bp, it is unable to confer estrogen regulation alone and must cooperate with other regulatory elements (54). In the case of the PR promoter, only a half-site ERE is found, and estrogen induction of PR appears to require cooperation with nearby Sp1 and AP-1 sites (55). Based on the obser-

vation that ERE-Vit-CAT (Fig. 1B) and ERE-pS2-Luc (Fig. 2) activities correlate with cellular concentrations of ERα, we suggest that ERα levels are the determining factor for the transcription activity of genes controlled exclusively by ERE. We further suggest that transcriptional activity of endogenous genes driven predominantly by an ERE (*e.g.* pS2) may depend upon the availability of ERα. In contrast, the level of ERα is unlikely to be the sole determining factor for the transcription of genes without a consensus ERE in their complex promoters (*e.g.* PR). In support of this notion, it has been reported that E2-induced transcription of the PR gene does not parallel ERα occupancy (55). Therefore, it is possible that MG132 inhibits PR expression through other protein factors, either directly or indirectly. In this respect, when evaluating the transcriptional activity of ERα, after escaping proteasome degradation, promoter context must be considered. Based on our own and the results of others (50), it is plausible that the transcription rate of a gene driven predominantly by an ERE is a more reliable readout of ERα transcription activity than a gene containing a complex promoter requiring ERα plus other transcription factors.

Our results differ from a previous study by Reid *et al.* (35), showing that MG132 prevented recruitment of phosphorylated RNA pol II (p-Pol II) to the pS2 promoter. This is most likely due to different experimental conditions and endpoints used in the two studies. For example, in their study Reid *et al.* used a higher dose (10 μM) and longer pretreatment (7 h) with MG132. However, under that condition, it is not clear whether the drug had any effect on p-Pol II recruitment to non-estrogen-responsive promoters. In addition, although α-amantin was used to clean the pS2 promoter before p-Pol II recruitment analysis, it is not clear that gene transcription resumed immediately (within a 2 h period) after α-amantin treatment. Thus, whether the differential recruitment of p-Pol II, in the absence or presence of MG132 after α-amantin pretreatment, is correlated with pS2 gene transcription remains an open question. However, the observation by Reid *et al.* (35) that the 20S proteolytic subunit does not associate with the pS2 promoter in response to E2 stimulation, agrees with numerous studies showing that the 20S proteasome subunit is not required for transcription initiation and elongation (56–60). Our observation further shows that 20S proteasome activity is not essential for ERα-mediated gene transcription.

Although the mechanism(s) by which the proteasome modulates ERα-mediated transactivation remains to be fully elucidated, chromatin immunoprecipitation assays have demonstrated that both unliganded and liganded receptors constantly cycle on and off estrogen-responsive promoters (35). MG132 appears to halt this cyclic interaction, leading to prolonged occupancy of ERα on EREs (35). The cyclic turnover of ERα could be a mechanism used by cells to prevent multiple rounds of transcription initiation from a single promoter, thus ensuring an appropriate cellular response to changes in circulating concentrations of hormone. To support this explanation, recent studies of GR show that proteasome inhibition dramatically increases both the residence time of GR on its target promoter and transcriptional output (24). In addition to extending the half-life of ligand-activated ERα, other factors, such as increased cellular concentration of receptor coactivators, could contribute to the enhancement of transcription by proteasome inhibition. Several ERα coactivators, including the steroid receptor coactivator family members (SRC1, SRC2, and SRC3) and cAMP response element binding protein (CREB)-binding protein/p300, are substrates of proteasomal degradation; proteasome inhibition appears to increase cellular concentrations of these coactivators (61).

We found that blocking ERα degradation (using MG132, Ubc12C111S, or UbK0) decreases E2-induced ERE-pS2-Luc expression at earlier time points (1.5–6 h) after E2 treatment (Figs. 3 and 4). Although the reason for this is unknown, one possibility is that ubiquitination and 20S proteasome activity are required for optimal ERα activation, perhaps by facilitating the release of ERα from preexisting corepressor complexes. To fully elucidate the physiological role(s) of ubiquitination, identification of the primary Ub ligase(s) for ERα, as well as the ubiquitination site(s) in this receptor, will be necessary.

In target tissues where ERα levels are limiting, the magnitude of the response to E2 is correlated with cellular ERα concentrations (2, 62). The Ub-proteasome pathway, by modulating receptor protein turnover, could play an important role in determining cellular responses to circulating E2 levels. Our results indicate that both the magnitude and duration of E2-induced gene transcription are limited by proteasome-mediated degradation of ERα; therefore, it seems reasonable to speculate that defects in ERα degradation could lead to enhanced cellular responsiveness to estrogens. In support of this possibility, it has been demonstrated that thyroid hormone and insulin, by blocking ligand-induced ERα degradation, can augment E2-stimulated cell proliferation (39, 63). Therefore, our future studies will examine the functional impact of proteasome-mediated ERα degradation on complex biological responses to estrogens, such as mammary gland development. In addition, aberrant ERα expression and estrogen responsiveness have been linked to breast tumor pathogenesis and development (64–66). Our previous studies demonstrate that blocking ERα degradation render breast cancer cells insensitive to the growth-inhibitory effects of ICI 182,780, a potent ERα down-regulator (42). Whether defects in the ERα degradation pathway contribute to deregulated estrogen signaling in breast cancer cells and play a role in disease progression to antiestrogen resistance remains to be elucidated.

MATERIALS AND METHODS

Plasmid Construction

The construction of pSG5-ERα(HEGO), ERE2-pS2-Luc, pcDNA-HA-Ubc12C111S, C3T1-Luc, pcDNA-SRC1, pCS2-UbK0, and ERE-Vit-CAT has been described previously (43, 67, 68).

Cell Lines

The human cervical carcinoma cell line HeLa and the breast cancer cell line MCF-7 were purchased from ATCC (Manassas, VA). The ERα-negative endometrial Ishikawa cell line was kindly provided by Dr. S. Hyder (University of Missouri, Columbia, MO). HeLa and Ishikawa cells were maintained in MEM with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum. MCF7 cells were maintained in the same medium with the addition of 6 ng/ml insulin. Before experiments involving hormone treatment, cells were cultured in hormone-free medium (phenol red-free MEM with 3% dextran-coated charcoal-stripped fetal bovine serum) for 3 d.

Transient Transfection and Reporter Enzyme Assays

Cells (80% confluence) were transfected with an equal amount of total plasmid DNA (adjusted by corresponding empty vectors) by using LipofectAMINE Plus Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. The DNA/LipofectAMINE mixture was removed 5 h later and cells were placed in hormone-free medium. Unless stated otherwise, 24 h after transfection, cells were treated with vehicle (DMSO) or MG132 (Sigma Chemical Co., St. Louis, MO) for 1 h before E2 (Sigma) treatment. At the end of the experiment, cell lysates were prepared for reporter enzyme assays. Luciferase activity was determined using the Luciferase Assay System (Promega Corp., Madison, WI), Gal activity was determined using a chemiluminescent reporter assay (PE Applied Biosystems, Foster City, CA), and CAT activity was determined using the colorimetric CAT ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN). Total cellular protein was determined by using the Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA). Reporter activities were expressed as relative light units normalized to total cellular protein.

Q-PCR

MCF7 cells were plated at a density of 3×10^6 per 10-cm dish and allowed to grow in hormone-free medium for 3 d. The cells were pretreated with MG132 (5 μM) for 1 h before E2 (10 nM) treatment. Total RNA was prepared by a RNAeasy Mini Kit (QIAGEN, Valencia, CA), according to the manufacturer's protocol. RNA (2 μg) was reverse transcribed in a total volume of 40 μl containing 400 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (New England Biolabs, Beverly, MA), 400 ng random hexamers (Promega), 80 U RNase Inhibitor, and 1 mM deoxynucleotide triphosphates. The resulting cDNA was used in subsequent Q-PCR reactions, performed in 1× iQ SYBR Green Supermix (Bio-Rad) with 5 pmol forward and reverse primers. The primers used in the Q-PCR were, for pS2 mRNA: forward primer, 5′-ATACCATCGACGTCCCTCCA-3′; and reverse primer, 5′-AAGCGTGTCTGAGGTGTCCG-3′ (69); for pS2 hnRNA: forward primer, 5′-TTGGAGAAGGAAGCTGGATGG-3′ (start position 3997, within the intron); reverse primer, 5′-ACCACAATTCTGTCTTTCACGG-3′ (start position 4126, within the second exon); for PR: forward primer, 5′-TCAGTGGGCAGATGC TGTATTT-3′; and reverse primer, 5′-GCCACATGGTAAGGCATAATGA-3′ (70); for cathepsin D: forward primer, 5′-GTACATGATCCCCTGTGAGAAGGT-3′; reverse primer, 5′-GGGACAGCTTGTAGCCTTTGC-3′ (71); and for β-actin: forward primer, 5′-TGCGTGACATTAAGGAGAAG-3′; and reverse primer, 5′-GCTCGTAGCT CTTCTCCA-3′. Q-PCR was performed in 96-well optical plates (Bio-Rad) using an iCycler system (Bio-Rad) for 40 cycles (94 C for 10 sec, 60 C for 40 sec), after an initial 3-min denaturation at 94 C. The relative concentration of RNA was calculated using the ΔΔCt method according to Relative Quantitation of Gene Expression (Applied Biosystems User Bulletin) with β-actin mRNA as an internal control. Results were expressed as relative RNA levels standardized such that values obtained in cells treated with vehicle (DMSO) only were set to 1.

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Address all correspondence and requests for reprints to: Kenneth P. Nephew, Ph.D., Medical Sciences, Indiana University School of Medicine, 302 Jordan Hall, 1001 East 3rd Street, Bloomington, Indiana 47405-4401. E-mail: knephew@indiana. edu.

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APPENDIX 3

CHIP (Carboxyl Terminus of Hsc70-Interacting Protein) Promotes Basal and Geldanamycin-Induced Degradation of Estrogen Receptor-alpha

Meiyun Fan[1], Annie Park[1], and Kenneth P. Nephew[1,2,3,4]

[1]Medical Sciences, Indiana University School of Medicine, Bloomington, IN 47405; [2]Indiana University Cancer Center; [3]Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN 46202.

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[4]To whom correspondence should be addressed:
Kenneth P. Nephew, Ph.D.
Medical Sciences
Indiana University School of Medicine
302 Jordan Hall
1001 E. 3rd St.
Bloomington, Indiana 47405-4401
Phone: (812) 855-9445
FAX: (812) 855-4436
E-mail:knephew@indiana.edu

Abbreviations: CHIP, carboxyl terminus of Hsc70-interacting protein; csFBS, dextran-coated charcoal-stripped fetal bovine serum; DMSO, Methyl sulfoxide; E2, 17β-estradiol; ERα, estrogen receptor-alpha; ERE, estrogen response element; GA, geldanamycin; GFP, green fluorescence protein; HA, hemagglutinin; Hsp, heat shock protein; ICI, ICI 182,780; Luc, firefly luciferase; OHT, 4-hydroxytamoxifen; siRNA, small interference RNA; TPR, tetratricopeptide repeat.

Key Words: estrogen receptor, CHIP, geldanamycin, ubiquitin, Hsp90

ABSTRACT

In estrogen target cells, estrogen receptor-alpha (ERα) protein levels are strictly regulated. Although receptor turnover is a continuous process, dynamic fluctuations in receptor levels, mediated primarily by the ubiquitin-proteasome pathway, occur in response to changing cellular conditions. In the absence of ligand, ERα is sequestered within a stable chaperone protein complex consisting of heat shock protein 90 (Hsp90) and co-chaperones. However, the molecular mechanism(s) regulating ERα stability and turnover remain undefined. One potential mechanism involves CHIP, the carboxyl terminus of Hsc70-interacting protein, previously shown to target Hsp90 interacting proteins for ubiquitination and proteasomal degradation. In the present study, a role for CHIP in ERα protein degradation was investigated. In ER-negative HeLa cells transfected with ERα and CHIP, ERα proteasomal degradation increased, while ERα-mediated gene transcription decreased. In contrast, CHIP depletion by siRNA resulted in increased ERα accumulation and reporter gene transactivation. Transfection of mutant CHIP constructs demonstrated that both the U-box (containing ubiquitin ligase activity) and the tetratricopeptide repeat (TPR, essential for chaperone binding) domains within CHIP are required for CHIP-mediated ERα downregulation. In addition, coimmunoprecipitation assays demonstrated that ERα and CHIP associate through the CHIP TPR domain. In ERα-positive breast cancer MCF7 cells, CHIP overexpression resulted in decreased levels of endogenous ERα protein and attenuation of ERα-mediated gene expression. Furthermore, ERα-CHIP interaction was stimulated by the Hsp90 inhibitor geldanamycin (GA), resulting in enhanced ERα degradation; this GA effect was further augmented by CHIP overexpression, but was abolished by CHIP depletion. Finally, ERα dissociation from CHIP by various ERα ligands, including E2,

OHT, and ICI, interrupted CHIP-mediated ERα degradation. These results demonstrate a role for CHIP in both basal and GA-induced ERα degradation. Furthermore, based on our observations that CHIP promotes ERα degradation and attenuates receptor-mediated gene transcription, we suggest that CHIP, by modulating ERα stability, contributes to the regulation of functional receptor levels, and thus hormone responsiveness, in estrogen target cells.

INTRODUCTION

The primary mediators of 17β-estradiol (E2) action, the major female sex steroid hormone, are the estrogen receptors ERα and ERβ. These receptors function as ligand-activated transcription factors, regulating expression of genes coordinating most physiological and many pathophysiological processes in estrogen target tissues (1). Tissue sensitivity, and the overall magnitude of response to E2 and other estrogens, is strongly influenced by a combination of factors, including cellular levels of ERα and its various coactivators and corepressors (2, 3).

To strictly control cellular responses, the cellular synthesis and turnover of the ERα protein dynamically fluctuates with changing cellular environments (4). For example, in the absence of ligand, ERα is a short-lived protein (half-life of 4-5 hours) and undergoes constant degradation (5). In the presence of ligand, by contrast, the turnover rate of ERα can be increased or decreased, depending upon the ligand, thus modulating receptor protein levels. Turnover-inducing factors and conditions include the cognate ligand E2, pure antiestrogens (ICI 164,384, ICI 182,780, RU 58,668), Hsp90 inhibitors (geldanamycin and radicicol), ATP depletion (oligomycin and hypoxia) and aryl hydrocarbon agonists; these all induce degradation and rapid downregulation of ERα levels (6-12). In contrast, the partial agonist/antagonist 4-hydoxytamoxifen (OHT), thyroid hormone, and protein kinase K activators (Forskolin, 8-bromo-cAMP) all block receptor degradation , subsequently increasing ERα protein levels (13-15).

Although both basal and ligand-induced ERα degradation are mediated by the ubiquitin-proteasome pathway (12, 13, 16-21), regulation of this pathway, at the molecular level, remains unclear. Emerging evidence suggests that multiple ERα degradation pathways exist, and the engagement of one pathway over another depends on the nature of the stimulus (19, 21-23). For example, E2-induced receptor degradation is coupled with transcription and requires new protein

synthesis (17, 19, 22, 24); conversely, neither ERα transcriptional activity nor new protein synthesis are needed for ICI 182,780-induced ERα degradation (19, 20, 22). In addition, various stimuli induce distinct changes in the conformation and cellular compartmentalization of ERα, these likely are associated with receptor ubiquitination (22, 25-27).

Like other members of the steroid receptor superfamily, unliganded ERα, by associating with various Hsp90-based chaperone complexes, is maintained in a ligand-binding competent conformation (28). Although these associations do not influence ERα ligand-binding affinity, Hsp90 chaperone complexes appear to regulate ERα stability, as Hsp90 disruption induces rapid ERα degradation through the ubiquitin proteasome pathway (9, 28, 29). For regulation of such complexes, recent studies have identified the carboxyl terminus of Hsc70-interacting protein (CHIP) as a ubiquitin ligase that directs chaperone substrates for ubiquitination and proteasomal degradation (30, 31). CHIP interacts with Hsp/Hsc70 and Hsp90 through an amino-terminal tetratricopeptide repeat (TPR) domain and catalyzes ubiquitin conjugation through a carboxyl-terminal U-box domain (30). As recent observations demonstrate that CHIP targets a number of Hsp70/90-associated proteins for ubiquitination and degradation, including the glucocorticoid receptor, androgen receptor, Smad1/4, and ErbB2 (30-33), we investigated a regulatory role for CHIP in ERα stability. Our results demonstrate that CHIP, likely through a chaperone intermediate, associates with ERα and consequently facilitates both basal and geldanamycin-induced receptor degradation in human cancer cells.

RESULTS

Overexpression of CHIP downregulates ERα protein levels

To investigate the effect of CHIP overexpression on steady state levels of ERα, ER-negative HeLa cells were cotransfected with constructs expressing CHIP (pcDNA-His6-CHIP) and ERα (pSG5-ERα) and ERα protein levels subsequently determined by immunoblot analysis. Overexpression of CHIP decreased ERα protein levels in a dose-dependent manner (Fig. 1A). To control for transfection efficiency, the green fluorescence protein (GFP) was also included in transfection. No effects of CHIP on GFP expression levels were observed (Fig. 1A), demonstrating that CHIP-induced downregulation of ERα was specific. To examine the effect of endogenous CHIP on ERα levels, we transfected HeLa cells with a CHIP-siRNA expressing construct, pBS/U6/CHIPi (33). In HeLa cells transfected with CHIP, expression of CHIP-siRNA dramatically decreased CHIP expression, but showed no effect on GFP level (Fig. 1B, *upper panel*), confirming that CHIP-siRNA specifically blocks CHIP expression. In cells transfected with ERα, expression of CHIP-siRNA elevated ERα protein level (Fig. 1B, *middle panel*). In addition, expression of CHIP-siRNA attenuated CHIP-induced ERα downregulation in a dose-dependent fashion (Fig. 1B, *lower panel*). Together, these results demonstrate that expression of CHIP protein alone can induce degradation of ERα and also indicate that ERα is continuously targeted for degradation by endogenous CHIP.

CHIP down-regulates ERα levels through the ubiquitin proteasome pathway

To determine whether proteasome activity is required for CHIP-induced ERα downregulation, HeLa cells were cotransfected with pcDNA-His6-CHIP and pSG5-ERα, treated with the proteosome inhibitor MG132, and subjected to immunoblotting. As shown in Fig. 2A,

6-hr treatment with MG132 completely blocked CHIP-induced downregulation of ERα. To examine whether polyubiqutination is required for CHIP-induced ERα degradation, a mutant ubiquitin, UbK0, with all lysines replaced by arginines (34), was utilized. Previously, we showed that the UbK0 protein could efficiently block E2-induced ERα degradation (35). Expression of UbK0, but not wild-type ubiquitin, restored ERα protein levels (Fig. 2B), demonstrating that CHIP stimulates ERα degradation through the ubiquitin and proteasome pathway.

CHIP targets mature ERα for degradation

It has been proposed that CHIP functions as a general ubiquitin ligase, responsible for ubiquitinating unfolded or misfolded proteins in a chaperone-dependent process (31). To examine whether ERα downregulation by CHIP was due to the selective ubiquitination of unfolded or misfolded receptor protein, we examined the effect of OHT, a selective estrogen receptor modulator, on CHIP-mediated ERα degradation. It has been shown that OHT can dissociate ERα from its chaperone complex and protect the receptor from both basal turnover and degradation induced by Hsp90-binding agents (8, 13, 21). We reasoned that if CHIP selectively targets immature or misfolded ERα (with no functional OHT-binding pocket), then, in the presence of CHIP, OHT treatment should not restore ERα levels. On the other hand, if CHIP targets mature ERα, OHT treatment should rescue the receptor protein from CHIP-induced degradation. HeLa cells were thus cotransfected with pcDNA-His6-CHIP and pSG5-ERα and treated with OHT for 6 h prior to lysate preparation. OHT treatment completely abolished CHIP-induced ERα downregulation (Fig. 2A), but had no effect on protein levels of CHIP and GFP, excluding the possibility that OHT treatment affects protein degradation in general. These

results demonstrate that CHIP induces degradation of correctly folded, ligand-binding competent ERα.

Both the TPR and U-box domains are essential for CHIP-induced ERα downregulation

To examine whether the ubiquitin ligase activity and chaperone interaction domain are required for CHIP-induced ERα degradation, two mutant CHIP constructs were utilized: 1) CHIP(K30A), a TPR domain mutant unable to interact with Hsp/Hsc70 or Hsp90; and 2) CHIP(H260Q), a U-box domain mutant unable to catalyze protein ubiquitin conjugation (36). In contrast to wild-type CHIP, neither CHIP(K30A) nor CHIP(H260Q) overexpression decreased ERα protein levels (Fig. 3A). These results establish that both the chaperone interaction and ubiquitin ligase activity of CHIP are required for CHIP-targeted degradation of ERα protein.

The TPR domain of CHIP is required for the CHIP-ERα interaction

As CHIP appears to be linked to ERα degradation, we investigated whether CHIP associates with the receptor. HeLa cells were cotransfected with ERα and CHIP, and coimmunoprecipitation analysis performed using an ERα-specific antibody. The results revealed a complex containing both CHIP and ERα (Fig. 3B). Because CHIP(K30A) exhibited no effect on ERα turnover (Fig. 3A), we examined whether the TPR domain is required for the CHIP-ERα interaction. In HeLa cells cotransfected with ERα and CHIP(K30A), the CHIP mutant was not detected in the precipitated ERα complex (Fig. 3B), demonstrating a requirement for the TPR domain in the CHIP-ERα interaction. Because it is known that CHIP interacts with Hsp90 or Hsc/Hsp70 through the TPR domain (30), our results suggest that a chaperone intermediate is involved in CHIP-induced ERα degradation.

CHIP interacts with endogenous ERα, in breast cancer cells, to induce receptor ubiquitination and degradation

Having demonstrated a role for CHIP (possibly in association with chaperones) in degradation of exogenous ERα in HeLa cells, we next examined the effect of CHIP on endogenous ERα levels. In human breast cancer MCF7 cells, overexpression of CHIP resulted in a dose-dependent ERα downregulation (Fig. 4A). Coimmunoprecipitation analysis of MCF7 cells transfected with pcDNA-His6-CHIP revealed both CHIP and ERα in the immunocomplexes precipitated by either an ERα-specific or anti-His6 antibody (Fig. 4B), suggesting that CHIP associates with endogenous ERα. Based on previous observations that CHIP can bind either Hsp/Hsc70 or Hsp90 (31), and that the TPR domain is essential for CHIP-ERα interaction (Fig. 3), we invested the possibility that CHIP targets ERα for degradation by associating with a chaperone-receptor complex. The association of Hsc70 and Hsp90 with ERα, in the presence or absence of overexpressed CHIP, was thus examined in MCF7 cells. Both Hsc70 and Hsp90 were detected in the precipitated ERα complex (Fig. 4B). However, CHIP overexpression slightly (but reproducibly) increased the level of Hsc70, but not Hsp90, in the ERα complex. These results indicate that CHIP may act to remodel the ERα chaperone complex to favor receptor degradation.

To determine whether CHIP promotes polyubiquitination of endogenous ERα, we examined the ubiquitination status of ERα in MCF7 cells transfected with hemaglutinin-tagged ubiquitin (HA-Ub), plus a vector control (pcDNA) or a CHIP expression construct. To block proteasomal degradation of polyubiquitinated proteins, transfected cells were treated with MG132 for 6 h prior to lysate preparation. An ERα-specific antibody was then used for immunoprecipitation, and the presence of ubiquitinated ERα in the immunocomplex detected by

immunoblotting with an HA antibody. To assess overall levels of protein ubiquitination, whole cell lysates were immunoblotted using an HA antibody. The polyubiquitinated ERα exhibited a typical high molecular weight smear on the blot membrane, and overexpression of CHIP markedly increased smear intensity, suggesting elevated receptor polyubiquination (Fig. 4C, *upper panel*). In contrast, CHIP had no effect on overall protein ubiquitination (Fig. 4C, *lower panel*). These results confirm that CHIP, by facilitating receptor polyubiquitination, targets endogenous ERα for proteasome-mediated degradation.

CHIP downregulates ERα-mediated reporter gene expression

Having established a role for CHIP in ERα ubiquitination and receptor turnover, we next examined the effect of CHIP on ERα-mediated gene transactivation. HeLa cells were transiently transfected with ERα and an estrogen responsive reporter (ERE-pS2-Luc), plus various CHIP (CHIP, H260Q, K30A, CHIPsiRNA) or control (pcDNA) constructs. Twenty-four hours after transfection, cells were treated for 6 h with vehicle (DMSO) or E2 (10 nM) and luciferase activity then measured. In a parallel experiment, a constitutive reporter (SV40-Luc) was used to monitor transcription efficiency, as well as any general effects of the various CHIP constructs might have on luciferase expression. The ERE-pS2-Luc activities were then normalized to the corresponding SV40-Luc activities. Expression of wild type CHIP decreased ($P<0.05$) E2-induced ERE-pS2-Luc expression, while the CHIP mutants had no effect on ERα-mediated gene transactivation (Fig. 5A). Conversely, CHIP depletion by siRNA increased both basal and E2-induced ERE-pS2-Luc expression (($P<0.01$, Fig. 5B). Similarly, in MCF7 cells, overexpression of CHIP, but not U-box or TPR mutant, attenuated ERα-mediated reporter gene expression (Fig. 6A), while CHIP depletion by siRNA augmented ERα-mediated gene expression (Fig. 6B).

Together, these results demonstrate that CHIP targets functional ERα for degradation and thereby coordinately regulates ERα protein levels and ERα-mediated gene transactivation

Geldanamycin induces ERα degradation through a CHIP-dependent mechanism

The Hsp90 inhibitor, geldanamycin (GA), binds to the amino-terminal ATP/ADP-binding domain of Hsp90, "locking" this chaperone protein in its ADP-bound conformation (9, 29, 37). CHIP has been reported to play a role in GA-induced degradation of ErbB2, a Hsp90 client protein (36, 38), and recent studies have shown that GA stimulates ERα degradation through the ubiquitin-proteasome pathway (9, 29, 37). Whether CHIP plays a role in GA-induced ERα degradation has not been previously investigated. Thus, we examined the effects of CHIP overexpression and depletion on GA-induced ERα degradation. In HeLa cells transfected with ERα, GA treatment resulted in a time-dependent ERα downregulation (Fig. 7A); this effect was enhanced by CHIP overexpression (Fig. 7A). Conversely, CHIP depletion by siRNA completely abolished GA-induced ERα downregulation (Fig. 7A).

To investigate the effect of GA on CHIP-ERα interaction, HeLa cells were transfected with ERα and CHIP, and coimmunoprecipition performed with an ERα-specific antibody. The amount of CHIP in the precipitated ERα complex increased following a 1-hr GA treatment (Fig. 7B), suggesting that GA promotes ERα degradation by recruiting CHIP to the chaperone-ERα complex. Because CHIP can associate with ubiquitinated proteins through its U-box domain (31), ERα ubiquitination may play a role in the GA-induced ERα-CHIP interaction. We thus examined the interaction between ERα and CHIP in the presence of the proteasome inhibitor MG132. We reasoned that, if CHIP preferentially interacts with ubiquitinated ERα, MG132, by

enhancing the accumulation of polyubiquitinated ERα, would increase the ERα-CHIP interaction. However, MG132 treatment did not increase the amount of CHIP precipitated with the ERα complex (Fig. 7B), suggesting that the GA-induced ERα-CHIP interaction occurs prior to ERα polyubiquitination.

To establish a role for CHIP in GA-induced ERα degradation under physiologically relevant conditions, the consequence of CHIP depletion by siRNA on ERα degradation was examined in GA-treated MCF7 cells. In MCF7 cells transfected with a pcDNA control plasmid, GA induced rapid ERα downregulation (Fig. 8A), consistent with previous reports (9, 29). However, transfection of CHIP-siRNA significantly impaired GA-induced ERα downregulation (Fig. 8A). In addition, enhanced association between endogenous CHIP and ERα was revealed by coimmunoprecipitation in MCF7 cells treated with GA (Fig. 8B). Based on these results, we suggest that CHIP is required for GA-induced ERα degradation.

Effects of ligand binding on geldanamycin-induced ERα degradation

Ligand binding results in disassembly of the ERα-Hsp90 chaperone complex, due to competition for overlapping binding sites and conformational changes within the ERα protein (28). Since GA stimulated the CHIP-ERα interaction (Fig. 7B and 8B), we investigated whether ligand binding, by interrupting the CHIP-ERα interaction, could interfere with GA-induced ERα degradation by examining ERα protein levels in MCF7 cells: 1) exposed to E2, ICI 182,780 (ICI) or GA alone; 2) pretreated with vehicle, E2, OHT or ICI for 30 min, followed by a 6-h treatment with GA; and 3) pretreated with vehicle or GA for 30 min, followed by a 5.5-hr treatment with E2, OHT or ICI. As expected, E2, ICI and GA treatment, but not OHT,

dramatically downregulated ERα levels in MCF7 cells (Fig. 9A, *upper panel*). Exposure to E2 or OHT, either prior to (Fig. 9A, *middle panel)* or short after (Fig. 9A, *lower panel)* GA treatment, completely abolished GA-induced ERα degradation. In contrast to what observed with E2 and OHT, ICI exposure, neither prior to (Fig. 9A, *middle panel)* nor short after (Fig. 9A, *lower panel)* GA treatment, failed to protect ERα against degradation.

To examine the effect of these ligands on the CHIP-ERα interaction, coimmunoprecipitation analysis was performed on MCF7 cells transfected with CHIP. Cells were pretreated with GA for 30 min, followed by a 30-min treatment with E2, OHT, or ICI. GA treatment alone increased the amount of CHIP detected in the precipitated ERα complex; however, this amount was substantially reduced by treatment with E2, OHT, or ICI (Fig. 9B). These results demonstrate that all three ligands can interfere with the interaction between CHIP and ERα. Because these ligands have dramatically different effects on ERα stability, our results indicate that after dissociation from the Hsp90 chaperone complex, distinct downstream pathways exist for ERα degradation. Because E2 alone can induce ERα degradation through a transcription coupled mechanism (17, 19, 22, 24), it was somewhat unexpected to observe that ERα was stable during the combined treatment of GA and E2 (Fig. 9A). One explanation is that Hsp90 activity (inhibited by GA) is required for transcription-coupled ERα degradation. The OHT-ERα complex lacks transcriptional activity in MCF7 cells and thus is not a substrate for the transcription-coupled degradation pathway. Consequently, the ability of OHT to block GA-induced ERα degradation was likely due to disruption of the CHIP-ERα interaction (Fig. 9B). ICI also interrupted the GA-induced CHIP-ERα interaction (Fig. 9B) but failed to stabilize ERα (Fig. 9A), suggesting that the ERα-ICI complex was targeted for degradation through a CHIP-independent, GA-insensitive pathway.

Effect of CHIP and geldanamycin on ERα cellular localization

CHIP and Hsp90 are located primarily in the cytoplasm (30), while ERα is primarily a nuclear-localized protein (39). To determine whether CHIP overexpression, or GA treatment, could affect the cellular distribution of ERα. HeLa cells were transfected with a GFP-ERα fusion protein (40) and the cellular distribution of green fluorescence examined. In control cells, fluorescence was restricted to the nuclei (Fig. 10A, *top left panel*). CHIP coexpression or GA treatment did not affect the nuclear localization of GFP-ERα (Fig. 10A). In contrast, ICI treatment, either alone or with transfected CHIP, resulted in the presence of green fluorescence in the cytoplasm (Fig. 10A, *bottom two panels*). This observation is consistent with a previous study by Dauvois *et al.* showing that ICI induces cytoplasmic retention of ERα (7). In addition, in HeLa cells transfected with GFP-ERα only, treatment with GA resulted in the appearance of GFP foci in the nuclei of approximately 20% of transfected cells (Fig. 10A, *left middle panel*). These GFP foci were not observed in GA-treated cells cotransfected with CHIP (Fig. 10A, *right middle panel*). While the identity of the GFP foci is unknown, one possibility is that these represent aggregated GFP-ERα, resulting from the combined effect of Hsp90 inhibition and high expression levels of GFP-ERα. CHIP overexpression may promote both basal and GA-induced ERα degradation, preventing GFP-ERα aggregate formation. Consistent with this interpretation, we found that expression of CHIP decreased the number of GFP-ERα-expressing cells (Fig. 10B). Based on our results, and a recent finding that a small fraction of nuclear-localized CHIP can promote nuclear protein degradation (41), we suggest that CHIP and GA-induced ERα degradation both occur within the nucleus.

DISCUSSION

The cellular level of ERα determines both estrogen sensitivity and responsiveness (2, 35, 42). Steady state levels of ERα protein are tightly regulated through a rapid balance between receptor synthesis and turnover, according to changing cellular conditions (4). Although it has been well documented that ERα degradation is primarily mediated by the ubiquitin proteasome pathway, the molecular mechanism(s) by which cells regulate ERα stability are largely unknown. Here we report that the Hsc70/Hsp90-interacting protein CHIP plays a key role in both basal and Hsp90 inhibitor-induced ERα turnover. Furthermore, CHIP-induced receptor degradation occurs through the ubiquitin proteasome pathway. Overexpression of CHIP promotes ERα degradation, accompanied by a decrease in ERα-mediated gene transactivation. Conversely, inhibition of CHIP by siRNA increases ERα levels and upregulates ERα-mediated gene transactivation. Thus, this is the first report that CHIP, by modulating the cellular concentration of ERα, directly coordinates estrogen action.

During the preparation of this report, Tateishi and colleagues reported a similar finding, that CHIP plays a role in basal ERα turnover (43). Our findings agree with several conclusions from that study, including: 1) CHIP, through its TPR domain, associates with ERα-chaperone complexes; 2) CHIP promotes, through its TPR and U-box domains, both polyubiquitination and proteasomal degradation of unliganded ERα; 3) CHIP-mediated ERα degradation occurs in the nucleus; and 4) ligand binding blocks CHIP-mediated ERα degradation by disrupting CHIP-ERα interaction. Here, we further extend the study of Tateishi *et al.* (43) in two significant aspects: 1) CHIP is required for Hsp90 inhibitor-induced ERα degradation; and 2) CHIP targets functional ERα for degradation (43).

Several lines of evidence from our study support the conclusion that CHIP targets functional ERα for degradation. First, OHT treatment completely blocked CHIP-induced ERα degradation, suggesting that ERα reaches a correctly folded conformation, competent for ligand binding, prior to CHIP-directed degradation. Secondly, CHIP overexpression downregulated ERα levels and decreased ERα-mediated gene expression, while CHIP depletion by siRNA upregulated ERα levels and increased ERα-mediated gene transcription. This coordinate regulation of ERα levels and activity suggests that CHIP targets functional ERα for degradation. Thirdly, CHIP plays a role in GA-induced ERα degradation by primarily targeting Hsp90-associated, transcriptionally competent ERα (29). Although originally believed to function as a general ubiquitin ligase, responsible for ubiquitinating unfolded or misfolded proteins in a chaperone-dependent process (31), more recent studies have demonstrated that CHIP also targets mature Hsp90 client proteins for degradation (33, 36).

Tateishi *et al.* (44) observed that CHIP overexpression increased ERα transcriptional activity. Although this was not observed in our study, the use of different estrogen-response element (ERE) and control reporter constructs for the functional analyses of ERα could account for this discrepancy. In the present study, an estrogen-responsive reporter construct (ERE-pS2-Luc), possessing two ERE copies within the pS2 promoter (44), was used. Our previous study demonstrated a close correlation between ERE-pS2-Luc expression and cellular concentration of ERα (35). In the present study, we also utilized a constitutively active construct, SV40-Luc, to monitor and normalize the effects of both CHIP and CHIP-siRNA on transfection efficiency and luciferase expression. In the study by Tateishi *el al.,* pRSVβGal was used as an internal control (44). When we used a similar construct, CMVβGal, we found that overexpression of either wild-type CHIP or TPR mutant (K30A), but not U-box mutant (H260Q), dramatically decreased

CMVβGal expression in a dose-dependent manner (data not shown). Based on these observations, we suggest that βGal is not a suitable control reporter for studying the effect of CHIP on gene transcription.

Our results, with data from Tateishi *et al.* (44), suggest a role for the Hsp90 chaperone complex in the regulation of cellular ERα levels. A summary of distinct ERα degradation pathways is depicted in Fig. 11. In the absence of activation signals, CHIP constantly targets chaperone-associated ERα for degradation, thereby limiting cellular concentrations of receptor protein. Ligand binding disassembles the ERα-Hsp90 complex and thus protects ERα from CHIP-mediated degradation. However, depending on the ligand, ERα stability can vary considerably, suggesting that different downstream destructive pathways exist. Furthermore, the ERα-ligand interaction could play a definitive role in pathway utilization. For example, when activated by E2, ERα is degraded through a transcription-coupled mechanism (17, 19, 22, 24). Pretreatment with GA, however, abolished E2-induced ERα degradation (Fig. 9A), suggesting that Hsp90 activity is required for transcription-coupled ERα degradation. In support of this possibility, the Hsp90-p23 complex has been shown to play a role in disassembling the nuclear receptor transcriptional complex from chromatin, a process believed to be a prerequisite for degradation of activated transcription factors (45-47). Conversely, through an unknown mechanism, the ERα-ICI complex is targeted for rapid degradation in MCF7 cells (48), in association with a cellular redistribution and aggregation of ERα (7, 8, 22, 27). Together, with our previous observation that an intact NEDD8 conjugation pathway is essential for ICI-induced ERα degradation in breast cancer cells (49), we suggest that destruction of the ICI-liganded receptor requires a cullin-based ubiquitin ligase.

Abnormal expression of ERα has long been associated with both the initiation and progression of breast cancer (50). An increase in the number of ERα-positive cells, as well as increased individual cell ERα content, have frequently been detected in malignant breast tumors (42). Furthermore, increased ERα content has been shown to augment the magnitude of estrogen-stimulated gene expression, providing a growth advantage to breast cancer cells (2, 35, 49, 51). A recent study demonstrated a correlation between the loss of ERK7, a regulator of estrogen-induced ERα degradation, and breast cancer progression (52). Collectively, these observations indicate that alterations in ERα degradation pathways may contribute to deregulation of ERα, perhaps leading to enhanced estrogen action in breast tumors. Based on our results, the chaperone/CHIP pathway, by regulating ERα levels, likely contributes to the development/progression of that disease; and such a possible role merits further examination.

MATERIALS AND METHODS

Materials

The following antibodies and reagents were used in this study: anti-ERα (HC20) and anti-β-tubulin (SC9104) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-HA tag (3F10; Roche Molecular Biochemicals, Indianapolis, IN); anti-ERα (Ab-10) and anti-GFP (GFP01) (NeoMarkers, Inc., Fremont, CA); anti-GAPDH (Chemicon International, Inc., Temecula, CA); anti-CHIP (PA1-015, Affinity Bioreagents, Golden, CO); anti-Hsp90 (SPA-830) and anti-Hsc70 (SPA-816) (Stressgene, Victoria, BC, Canada); anti-His6 (8906-1, BD Biosciences, Palo, Alto, CA); protein G-agarose beads (Oncogene Research Products, San Diego, CA); horseradish peroxidase-conjugated second antibodies and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL); protein assay kit (Bio-Rad laboratories Inc., Hercules, CA); protease inhibitor cocktail set III (Calbiochem-Novabiochem Corporation, San Diego, CA); LipofectAMINE Plus Reagent (Life Technologies, Inc., Logan, UT); FuGENE (Roche Molecular Biochemicals, Indianapolis, IN); 17β-estradiol, 4-hydroxytamoxifen, geldanamycin and MG132 (Sigma Chemical Co., St. Louis, MO); ICI 182,780 (Tocris Cookson Ltd., Ellisville, MO); Passive lysis buffer and luciferase assay system (Promega Corp., Medison, WI); fetal bovine serum (FBS) and dextran-coated charcoal-stripped FBS (csFBS) (Hyclone laboratories, Inc., Logan, Utah); cell culture supplementary reagents (Life Technologies, Inc.; Rockville, MD).

Plasmid Construction

The construction of pSG5-ERα(HEGO), ERE2-pS2-Luc, SV40-Luc, pcDNA-HA-Ub, pCS2-UbK0 and CMV-GFP have all been described previously (35). The pcDNA-His6-CHIP,

pcDNA-His6-CHIP(K30A), and pcDNA-CHIP(H260Q) constructs were kindly provided by Dr. Neckers and Cam Patterson (36), the pBS/U6/CHIPi construct by Dr. Chang (33), and the GFP-ERα construct by Dr. Stenoien (40).

Cell Lines and Transient Transfection

The human cervical carcinoma cell line HeLa and the breast cancer cell line MCF-7 were purchased from ATCC (Manassas, VA). HeLa cells were maintained in minimum essential medium (MEM) with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% FBS. MCF7 cells were maintained in the same medium, with the addition of 6 ng/ml insulin. Prior to experiments, cells were cultured in hormone-free medium (phenol red free MEM with 3% dextran-coated charcoal-stripped FBS (csFBS)) for 3 days. For transfection, cells (80% confluence) were transfected with an equal amount of total plasmid DNA (adjusted with the corresponding empty vectors) by using LipofectAMINE Plus Reagent or FuGENE according to the manufacturer's guidelines.

Immunoblotting, Immunoprecipitation and Luciferase Assay

For immunoblot analysis, whole cell extracts were prepared by suspending cells (~2 x 10^6) in 0.1 ml SDS lysis buffer (62 mM Tris, pH 6.8, 2% SDS, 10% Triton X-100, and protease inhibitor cocktail III). After 15 min incubation on ice, extracts were sonicated (3 x 20 sec), insoluble material removed by centrifugation (15 min at 12,000 x g), and supernatant protein concentration determined using a BioRad protein assay kit. Five percent β-mercaptoethanol was added to the protein extracts before heating at 90 ^{0}C for 5 min. Protein extracts (50 μg per lane) were

fractionated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and probed with antibodies. Primary antibody was detected by horseradish peroxidase-conjugated second antibody and visualized using an enhanced SuperSignal West Pico Chemiluminescent Substrate. The band density of exposed films was evaluated with ImageJ software (http://rsb.info.nih.gov/ij/). Immunoprecipitation was performed as described previously (49). For luciferase assays, cell lysates were prepared with passive lysis buffer and luciferase activity determined using the Luciferase Assay System.

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FIGURE LEGENDS

Fig. 1. Overexpression of CHIP downregulates ERα protein levels.

A. Overexpression of CHIP downregulates ERα protein levels. HeLa cells were transfected with 250 ng pSG5-ERα, 100 ng CMV-GFP, and various amounts (0, 50, 100 and 250 ng) of pcDNA-his6-CHIP. Protein levels of ERα, CHIP and GFP were determined by immunoblotting with anti-ERα, anti-His6 and anti-GFP, respectively. GFP expression was used as a control for transfection efficiency and SDS-PAGE loading. *B. Expression of CHIP-siRNA attenuates CHIP-induced ERα downregulation.* HeLa cells were transfected with various constructs (250 ng for each) as indicated, and protein levels were determined by immunoblot analysis. GAPDH was used as an SDS-PAGE loading control. For all experiments, 3 x 10^5 HeLa cells were plated in 60-mm dishes, cultured in hormone-free medium for 3 days, and then transfected with LipofectAMINE Plus Reagent. Cell lysates were prepared 24 hr after transfection. Representative results of two independent experiments, each performed in duplicate, are shown.

Fig. 2. The proteasome inhibitor MG132, partial ERα-antagonist OHT, and ubiquitin mutant UbK0, all block CHIP-induced ERα degradation.

A. The proteasome inhibitor MG132 and the partial ERα antagonist OHT block CHIP-induced ERα downregulation. HeLa cells were transfected with 250 ng pSG5-ERα and 100 ng CMV-GFP, along with 250 ng pcDNA (vector control) or pcDNA-His6-CHIP, then treated with DMSO (vehicle), 10 μM MG312 or 1 μM OHT for 6 hr prior to immunoblot analysis. Protein levels of ERα, CHIP and GFP were determined by immunoblotting with anti-ERα, anti-His6 and anti-GFP, respectively. GFP was used as a control for transfection efficiency and SDS-PAGE

loading. *B. Expression of the ubiquitin mutant UbK0 blocks CHIP-induced ERα downregulation.* HeLa cells were transfected with 250 ng pSG5-ERα, with or without 250 ng pcDNA-His6-CHIP, pcDNA-Ub or pCS2-UbK0, as indicated. ERα protein levels were determined by immunoblotting with anti-ERα. GAPDH was used as an SDS-PAGE loading control. For all experiments, $3x10^5$ HeLa cells were plated in 60-mm dishes, cultured in hormone-free medium for 3 days, and then transfected with LipofectAMINE Plus Reagent. Cell lysates were prepared 24 hr after transfection. The band density of exposed films was evaluated with ImageJ software. Relative ERα levels were presented as the mean ± SE of three independent experiments, each performed in duplicate.

Fig. 3. Both the TPR and U-box domains are required for CHIP-induced ERα downregulation. *A. Both the TRP and U-box domains are required for CHIP to downregulate ERα.* HeLa cells were transfected with 250 ng pSG5-ERα, 100 ng CMV-GFP, along with 250 pcDNA (control) or various CHIP constructs as indicated. ERα and GFP protein levels were determined by immunoblotting with anti-ERα and anti-GFP, respectively. GFP was used as control for transfection efficiency and SDS-PAGE loading. *B. The TRP domain is required for CHIP-ERα interaction.* HeLa cells were transfected with 250 ng pSG5-ERα, along with 250 ng pcDNA-His6-CHIP or pcDNA-His6-CHIP(K30A). ERα protein in cell lysates was precipitated with anti-ERα. The presence of CHIP in the precipitated ERα complex was determined by immunoblotting with anti-His6. The same blot was re-probed with anti-ERα to assess the amount of ERα in the precipitated immunocomplex. The expression levels of CHIP or CHIP(K30A) in whole cell lysates was determined by immunoblotting with anti-His6 (lower

panel). For all experiments, HeLa cells were plated in 60-mm dishes at a density of $3x10^5$ cells/dish, cultured in hormone-free medium for 3 days, and then transfected with LipofectAMINE Plus Reagent. Cell lysates were prepared 24 hr after transfection. Representative results of two independent experiments, each performed in duplicate, are shown.

Fig. 4. CHIP interacts with endogenous ERα and induces ERα ubiquitination and degradation in breast cancer MCF7 cells.

A. Overexpression of CHIP downregulates endogenous ERα levels in MCF7 cells. MCF7 cells were plated in 100-mm dishes at a density of $1x10^6$ cells/dish, cultured in hormone-free medium for 3 days, and transfected with various amounts (0, 5 or 10 μg) of pcDNA-His6-CHIP using FuGENE. Twenty four hours after transfection, whole cell lysates were prepared, and protein levels of ERα and CHIP determined by immunoblotting with anti-ERα and anti-His6, respectively. GAPDH was used as an SDS-PAGE loading control. *B. CHIP associates with ERα-Hsp complex in MCF7 cells.* MCF7 cells were transfected as in *A* and subjected to coimmunoprecipitation analysis. ERα and CHIP were precipitated with anti-ERα and anti-His6, respectively. The presence of CHIP, Hsc70, Hsp90 or ERα in the precipitated complexes was determined by immunoblotting with anti-His6, anti-Hsc70, anti-Hsp90, or anti-ERα, respectively. *C. Expression of CHIP enhances endogenous ERα polyubiquitination in MCF7 cells.* MCF7 cells were plated in 60-mm dishes at a density of $5x10^5$ cells/dish cultured in hormone-free medium for 3 days, and transfected with 250 ng pcDNA-HA-Ub and 250 ng pcDNA or pcDNA-His6-CHIP. Twenty four hours after transfection, whole cell lysates were prepared and ERα protein was precipitated with anti-ERα. The presence of ubiquitin-conjugated

ERα in the immunocomplex was detected by immunoblotting with anti-HA (upper panel). The same membrane was reprobed with anti-ERα to assess the amount of precipitated ERα (middle panel). Whole cell lysates were separated by SDS-PAGE and probed with HA antibody to determine the amount of total ubiquitinated proteins (lower panel). Representative results of two independent experiments, each performed in duplicate, are shown.

Fig. 5. CHIP downregulates ERα-mediated reporter gene expression in HeLa cells.

HeLa cells were plated in 12-well dishes at a density of $1x10^5$/well, grown in hormone-free medium for 3 days, and transfected with 10 ng pSG5-ERα, 250 ng ERE-pS2-Luc, 250 ng various CHIP constructs *(A)* or pBS/U6/CHIPi *(B)*. Twenty four hours after transfection, cells were treated for 6 hr with DMSO or 10 nM E2 and then assayed for luciferase activity. The ERE-pS2-Luc activity was normalized to SV40-Luc activity, which was determined in a parallel experiment where ERE-pS2-Luc was replaced with SV40-Luc. The results are expressed as means ± SE from three independent experiments, with each performed in quadruplicate. * $p<0.05$ (student's t-test, *vs* pcDNA treated with E2).

Fig. 6. CHIP downregulates ERα-mediated reporter gene expression in MCF7 cells.

MCF7 cells were plated in 12-well dishes at a density of $1x10^5$/well, grown in hormone-free medium for 3 days, and transfected with 250 ng ERE-pS2-Luc, along with 250 ng various CHIP constructs *(A)*, or various amounts (250, and 500 ng) of pBS/U6/CHIPi *(B)*. Twenty four hours after transfection, cells were treated for 6 hr with DMSO or 10 nM E2 and assayed for luciferase. The ERE-pS2-Luc activity was normalized to SV40-Luc activity, which was determined in a parallel experiment where ERE-pS2-Luc was replaced with SV40-Luc. The results were

expressed as mean ± SE from three independent experiments, each performed in quadruplicate. * $p<0.05$ (student's t-test, *vs* pcDNA treated with E2).

Fig. 7. Disruption of Hsp90 function induces ERα degradation through a CHIP-dependent mechanism in HeLa cells.

A. CHIP overexpression augments, while CHIP depletion by siRNA blocks, GA-induced ERα degradation. HeLa cells were plated in 60-mm dishes at a density of $3x10^5$ cells/dish, cultured in hormone-free medium for 3 days, and transfected with 250 ng pSG5-ERα, along with 250 ng pcDNA, pcDNA-His6-CHIP or pBS/U6/CHIPi by using LipofectAMINE Plus Reagent. Twenty four hours after transfection, the cells were treated with 1 μM GA for 0, 0.5, 1 and 3 hr. Cell lysates were immunoblotted with anti-ERα. GAPDH was used as an SDS-PAGE loading control. The band density of exposed films was evaluated with ImageJ software. Relative ERα levels were presented as mean ± SE from three independent experiments. *B. GA enhances CHIP-ERα interaction.* HeLa cells were plated as in *A* and transfected with 250 ng pSG5-ERα and 250 ng pcDNA-His6-CHIP. Twenty four hours after transfection, cells were untreated or treated with 1 μM GA or 10 mM MG132 for 1 hr prior to lysate preparation. ERα protein was precipitated by anti-ERα and the presence of CHIP determined by immunoblotting with anti-His6. The same membrane was then re-probed with anti-ERα to assess the amount of precipitated ERα in the same complex. Representative results of three independent experiments, each performed in duplicate, are shown.

Fig. 8. CHIP is required for geldanamycin-induced ERα degradation in breast cancer MCF7 cells.

A. CHIP depletion by CHIP-siRNA eliminates GA-induced ERα degradation. MCF7 cells were plated in 60-mm dishes at a density of $3x10^5$ cells/dish, cultured in hormone-free medium for 3 days, and transfected with 500 ng pcDNA (control) or pBS/U6/CHIPi. Twenty four hours after transfection, cells were treated with 1 μM GA for 0, 1, 2.5 and 4 hr, and subjected to immunoblotting with anti-ERα. β-tubulin was used as SDS-PAGE loading control. The band density of exposed films was evaluated with ImageJ software. Relative ERα levels are presented as mean ± SE from three independent experiments (lower panel). *B. GA stimulates CHIP-ERα interaction.* MCF7 cells were plated at $1x10^6$ cells in 100-mm dishes, cultured in hormone-free medium for 3 days, and treated with 1 μM GA for 0, 1 and 3 hr prior to lysate preparation. ERα protein was precipitated by anti-ERα and the presence of CHIP examined by immunoblotting with anti-CHIP. The same membrane was then reprobed with ERα antibody to assess the amount of precipitated ERα in the same complex. Representative results of two independent experiments, each performed in duplicate, are shown.

Fig. 9. Effect of ligand binding on geldenamycin-induced ERα degradation

A. ERα protein levels in MCF7 cells treated with GA before or after ligand exposure. MCF7 cells were plated in 60-mm dishes at a density of $3x10^5$ cells/dish and cultured in hormone-free medium for 3 days. *Upper panel*, cells were treated with vehicle, 10 nM E2, 1 μM OHT, 100 nM ICI or 1 μM GA for 6 hr; *Middle panel*, cells were exposed to indicated ligand for 30 min prior to a 6-hr GA treatment; *Lower panel*, 30 min after GA treatment, cells were exposed to

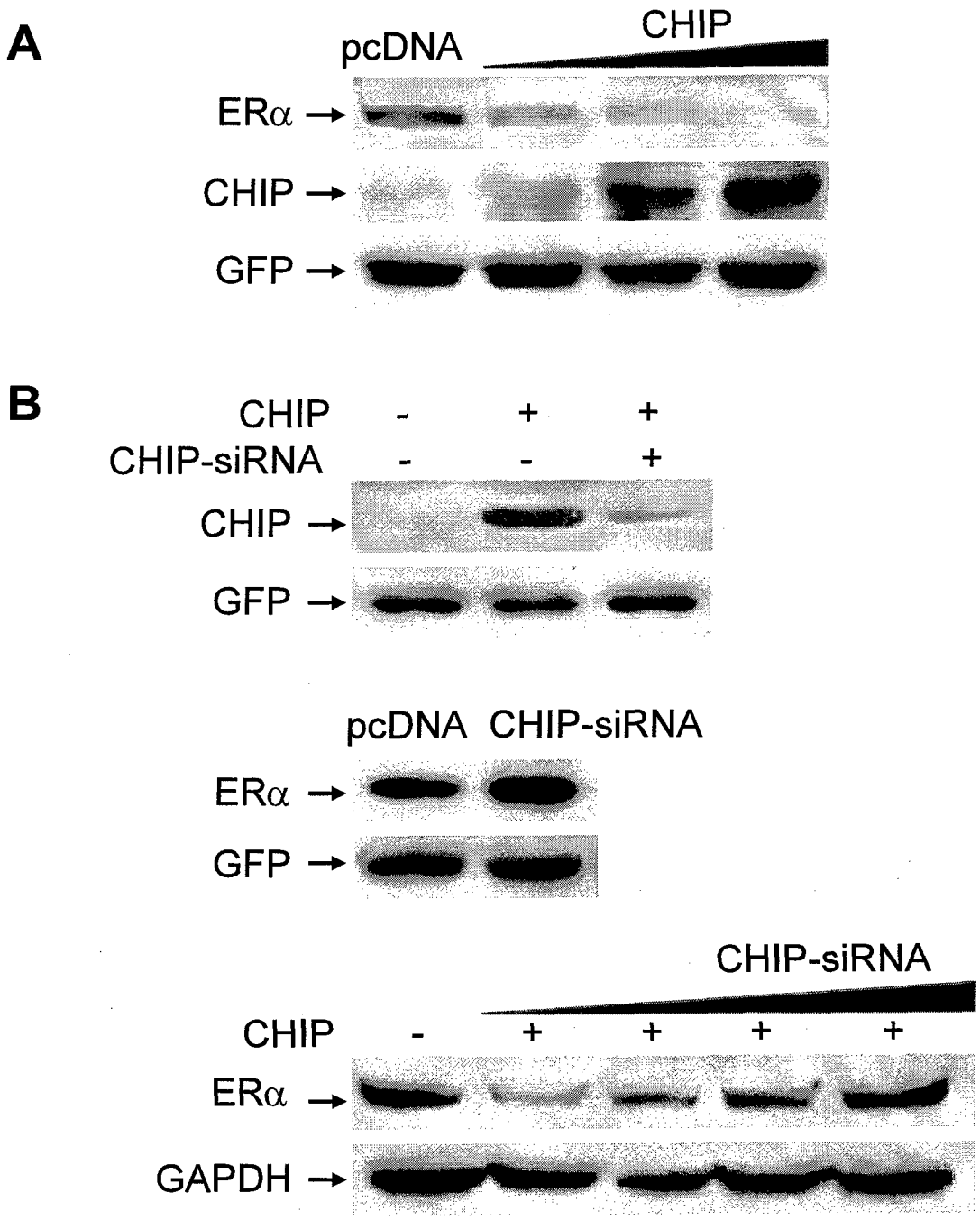
indicated ligand for 5.5 hr. For all experiments, ERα levels were determined by immunoblotting with anti-ERα. β-tubulin was used as SDS-PAGE loading control. B. *Effect of ligands on GA-induced CHIP-ERα interaction.* MCF7 cells were plated in 100-mm dishes at a density of $1x10^6$ cells/dish, cultured in hormone-free medium for 3 days, and then transfected with 5 μg pcDNA-His6-CHIP by using FuGENE. Twenty four hours after transfection, the cells were treated with 1 μM GA for 30 min, followed by a 30-min treatment with indicated ligands (100 nM E2, 1 μM OHT and 100 nM ICI). ERα protein from the cell lysates was precipitated using anti-ERα. CHIP presence in the precipitated ERα complex was determined by immunoblotting with anti-His6. The same membrane was re-probed with ERα antibody to assess the amount of precipitated ERα. Representative results of two independent experiments, each performed in duplicate, are shown.

Fig. 10. Effect of CHIP and geldanamycin on ERα cellular localization.

HeLa cells were plated in 6-well dishes at a density of $1x10^5$ cells/dish, cultured in hormone-free medium for 3 days, and transfected with 250 ng GFP-ERα and 250 ng pcDNA or pcDNA-His6-CHIP by using LipofectAMINE Plus Reagent. Twenty four hours after transfection, the transfected cells were treated with 1 μM GA or 100 nM ICI for 6 hr. The fluorescence of GFP-ERα was then examined using an inverted microscope (Axiovert 40 CFL) *(A)*. The number of cells expressing GFP-ERα from 10 microscope fields was present in the histogram *(B)*. Representative results of two independent experiments, each performed in triplicate, are shown.

Fig. 11. Schematic summary of distinct ERα degradation pathways.

In the absence of activation signals, CHIP constantly targets Hsp90-associated ERα for degradation. Ligand binding disassembles the ERα-Hsp90 complex and thus protects ERα from CHIP-mediated degradation. However, depending upon the ligand, distinct downstream destructive pathways are engaged in the degradation of liganded ERα. When activated by E2, ERα is degraded through a transcription-coupled mechanism. The ERα-ICI complex is targeted for a rapid degradation through a mechanism associated with cellular redistribution and aggregation of ERα. The OHT-ERα complexes are stable, likely due to the lack of transcriptional activity.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ERα
CHIP
GFP
B
CHIP - + +
CHIP-siRNA - - +
CHIP
GFP
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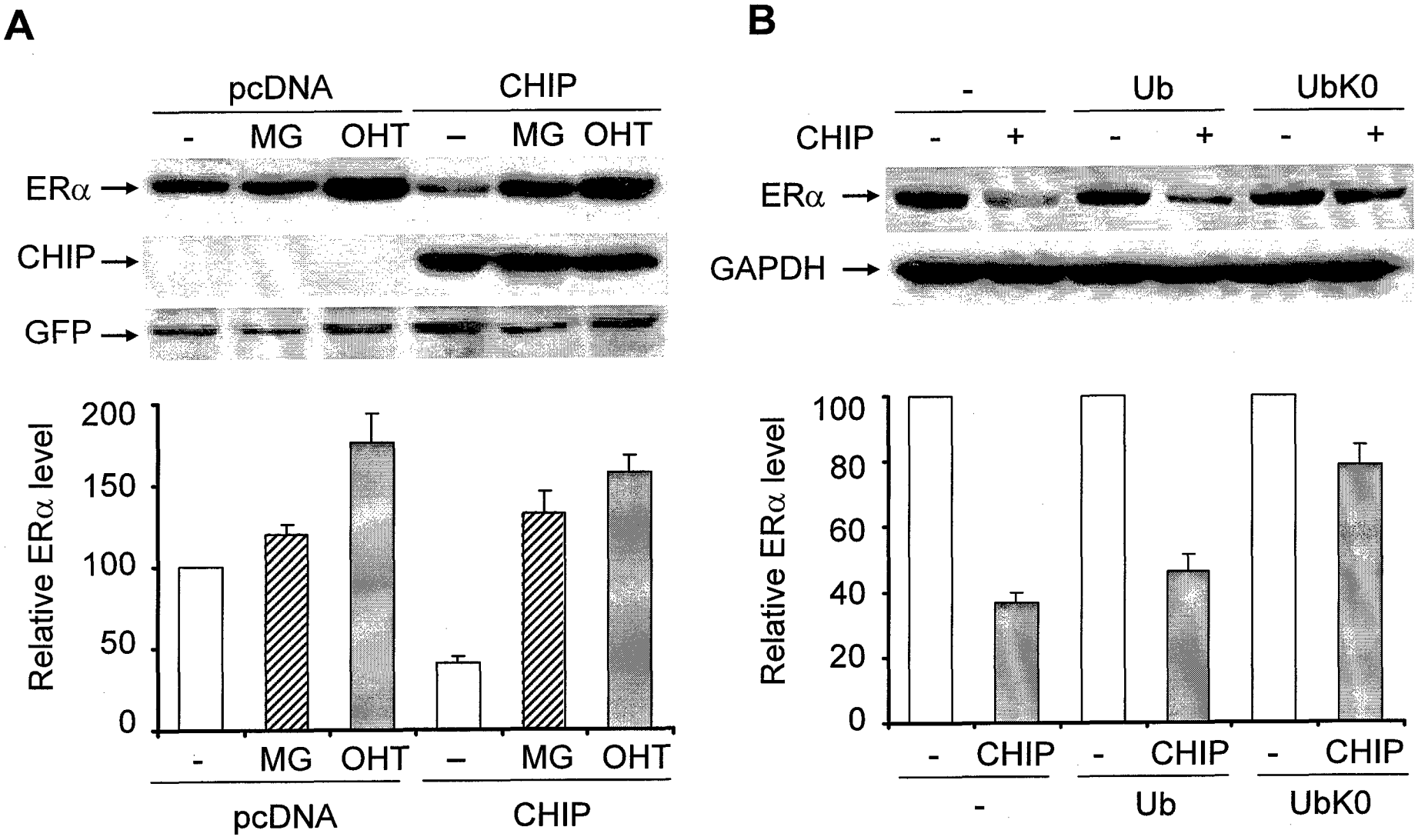
ERα
GFP
CHIP-siRNA
CHIP - + + + +
ERα
GAPDH

Fig. 1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GFP
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100
50
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pcDNA
CHIP
B
- Ub UbK0
CHIP - + - + - +
ERα
GAPDH
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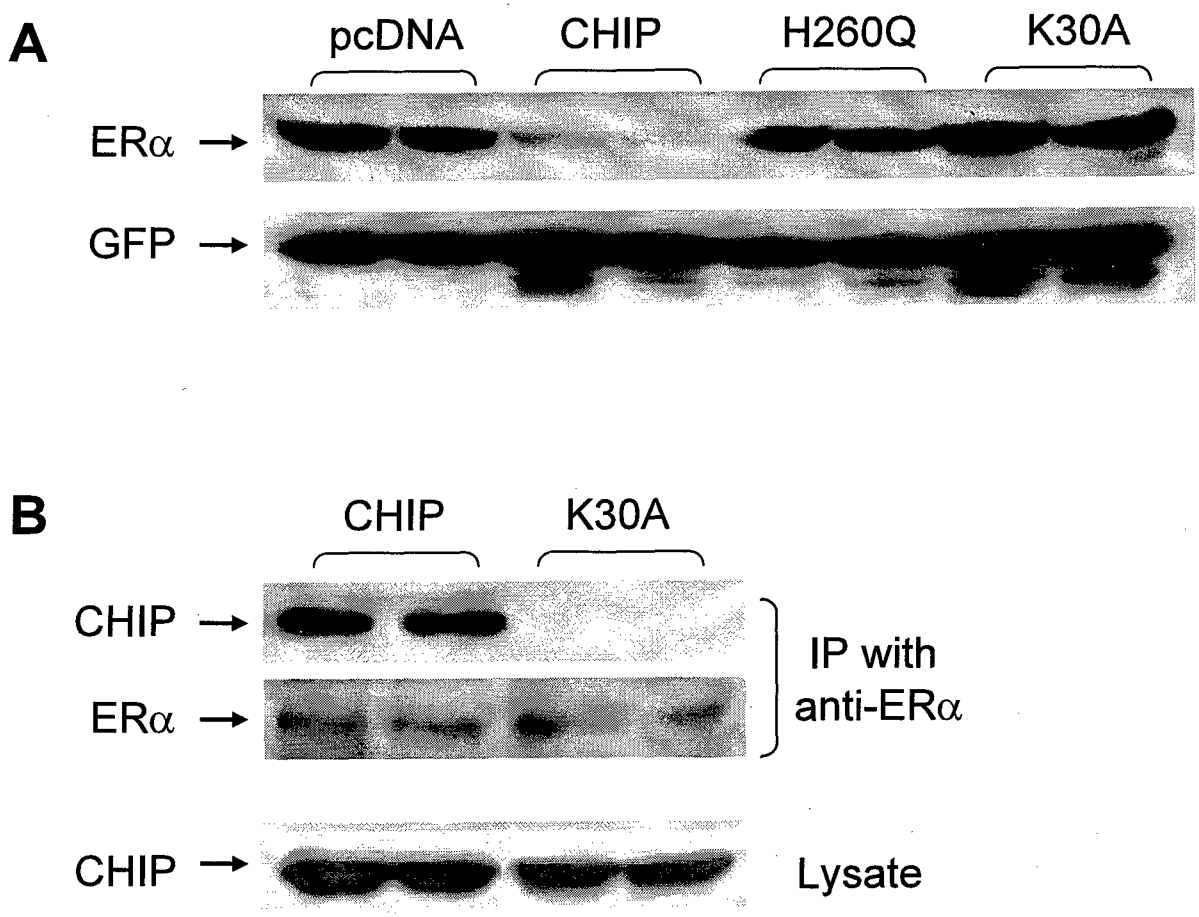
-
Ub
UbK0

Fig. 2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ERα
GFP
B
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K30A
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ERα
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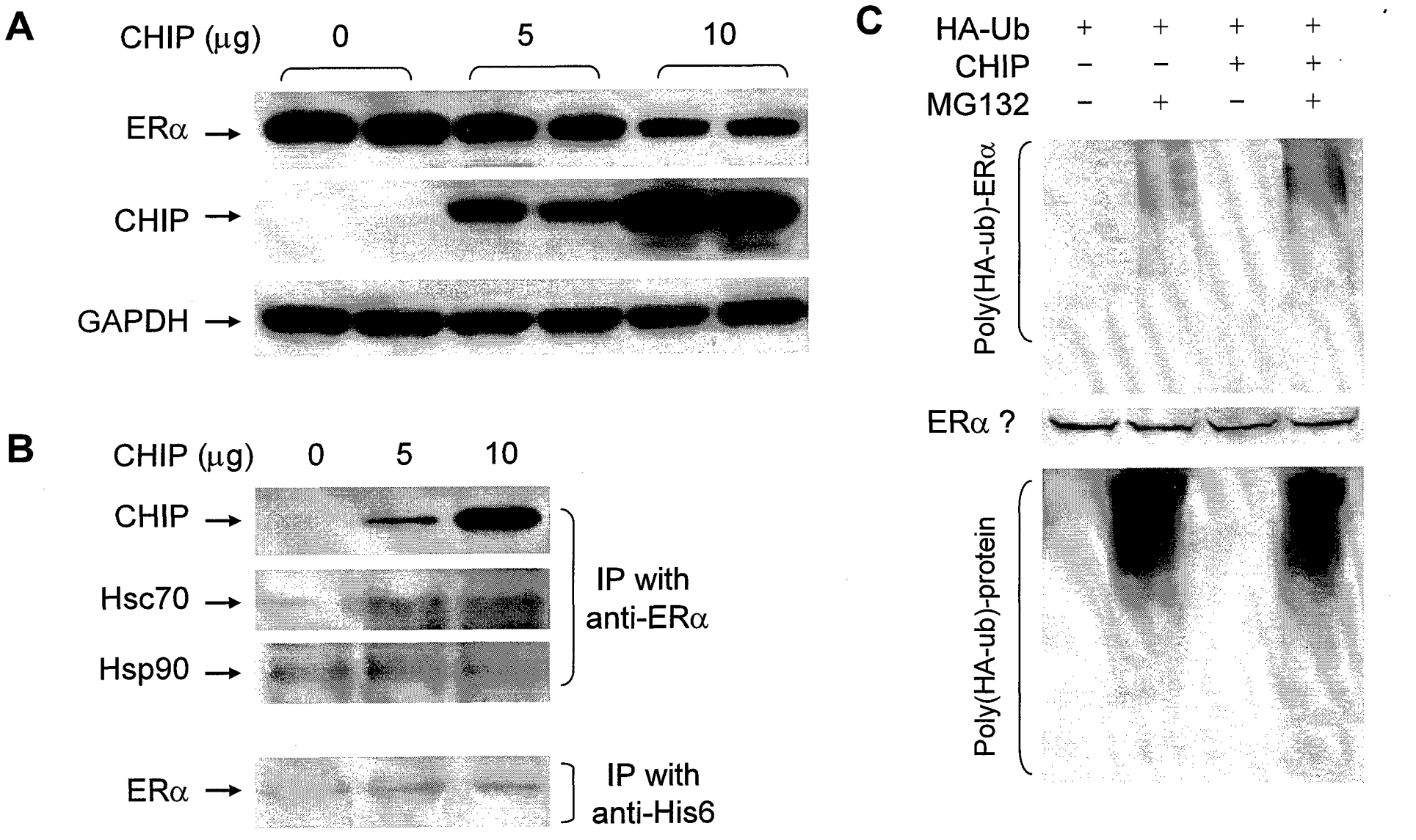
anti-ERα
CHIP
Lysate

Fig. 3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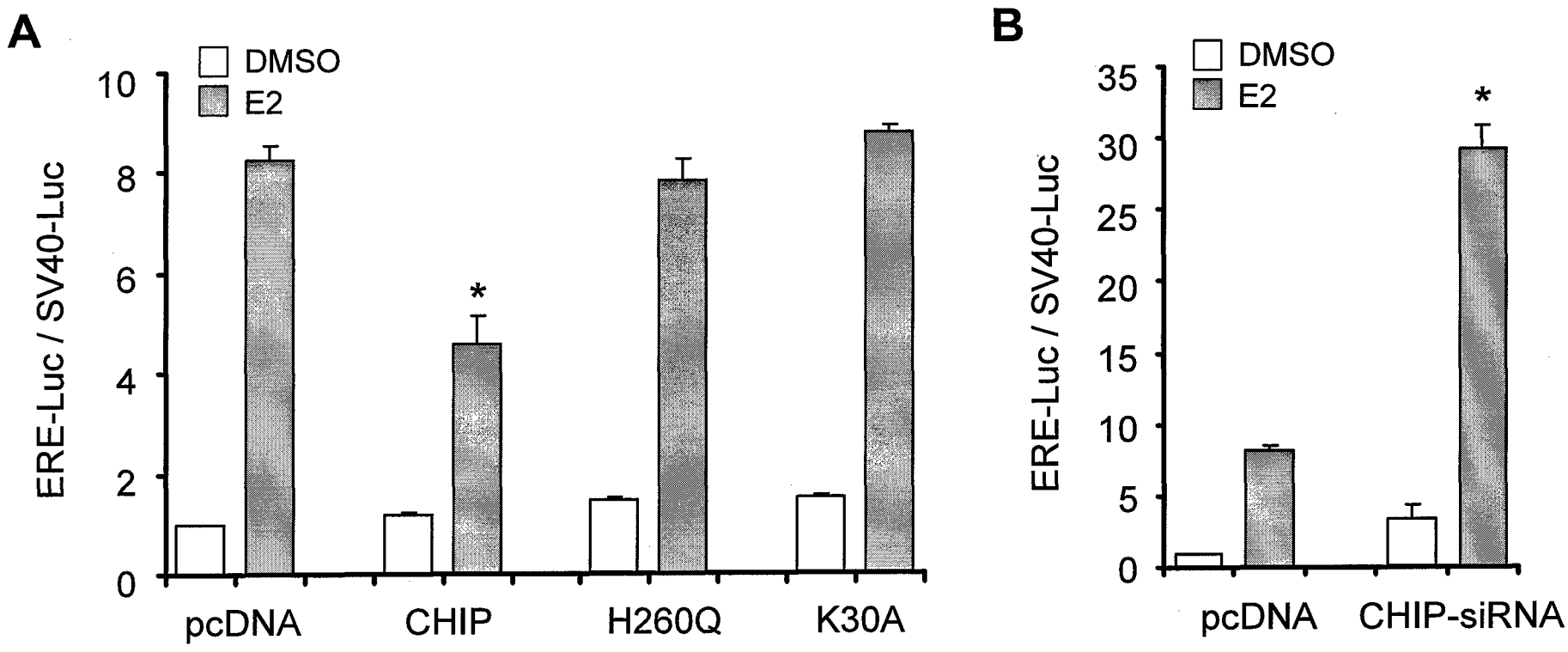
10
ERα
CHIP
GAPDH
B
CHIP (μg)
0
5
10
CHIP
Hsc70
Hsp90
IP with anti-ERα
ERα
IP with anti-His6
C
HA-Ub + + + +
CHIP − − + +
MG132 − + − +
Poly(HA-ub)-ERα
ERα ?
Poly(HA-ub)-protein

Fig. 4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5
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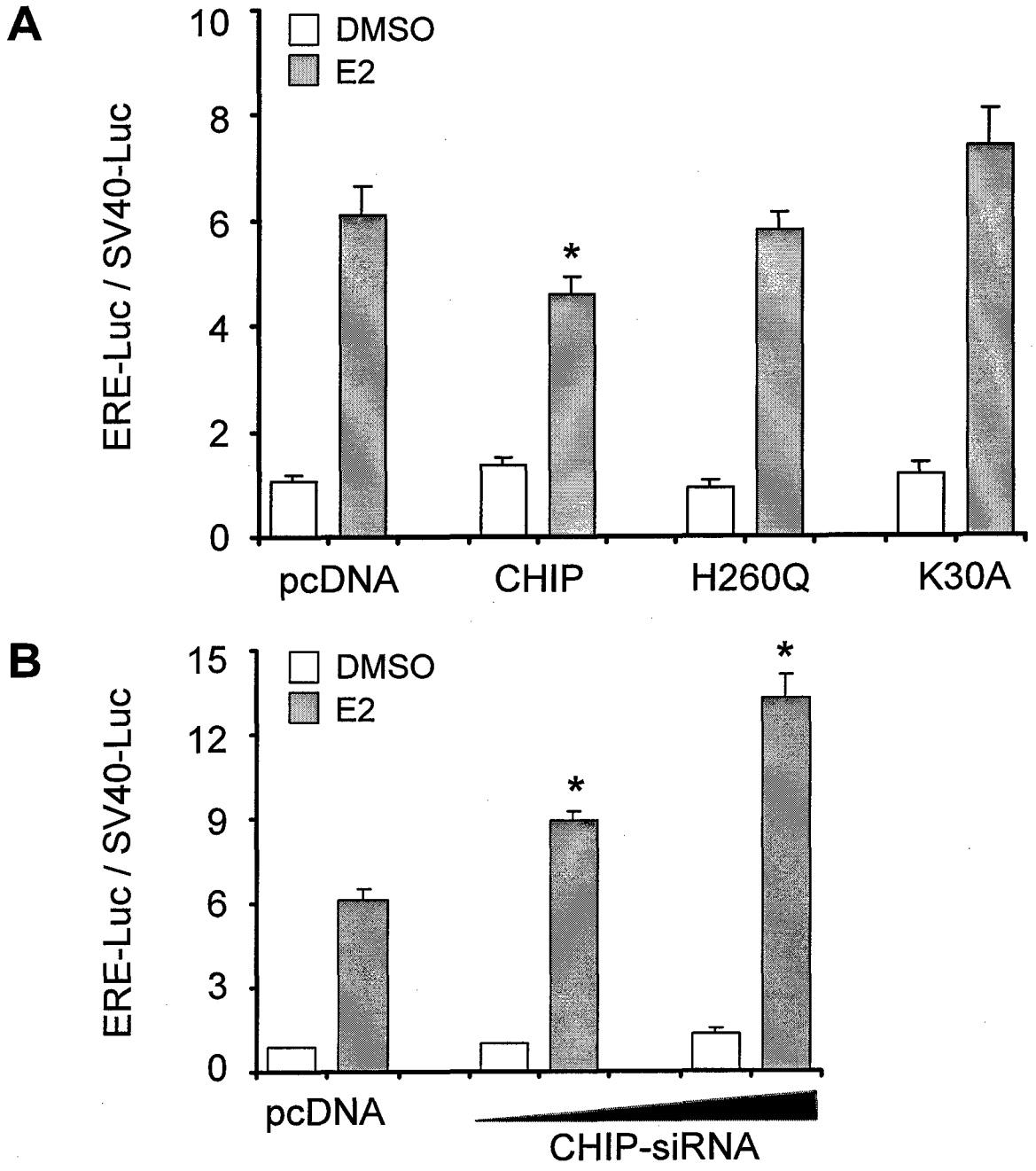
*
pcDNA
CHIP-siRNA

Fig. 5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ERE-Luc / SV40-Luc
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K30A
B
ERE-Luc / SV40-Luc
15
12
9
6
3
0
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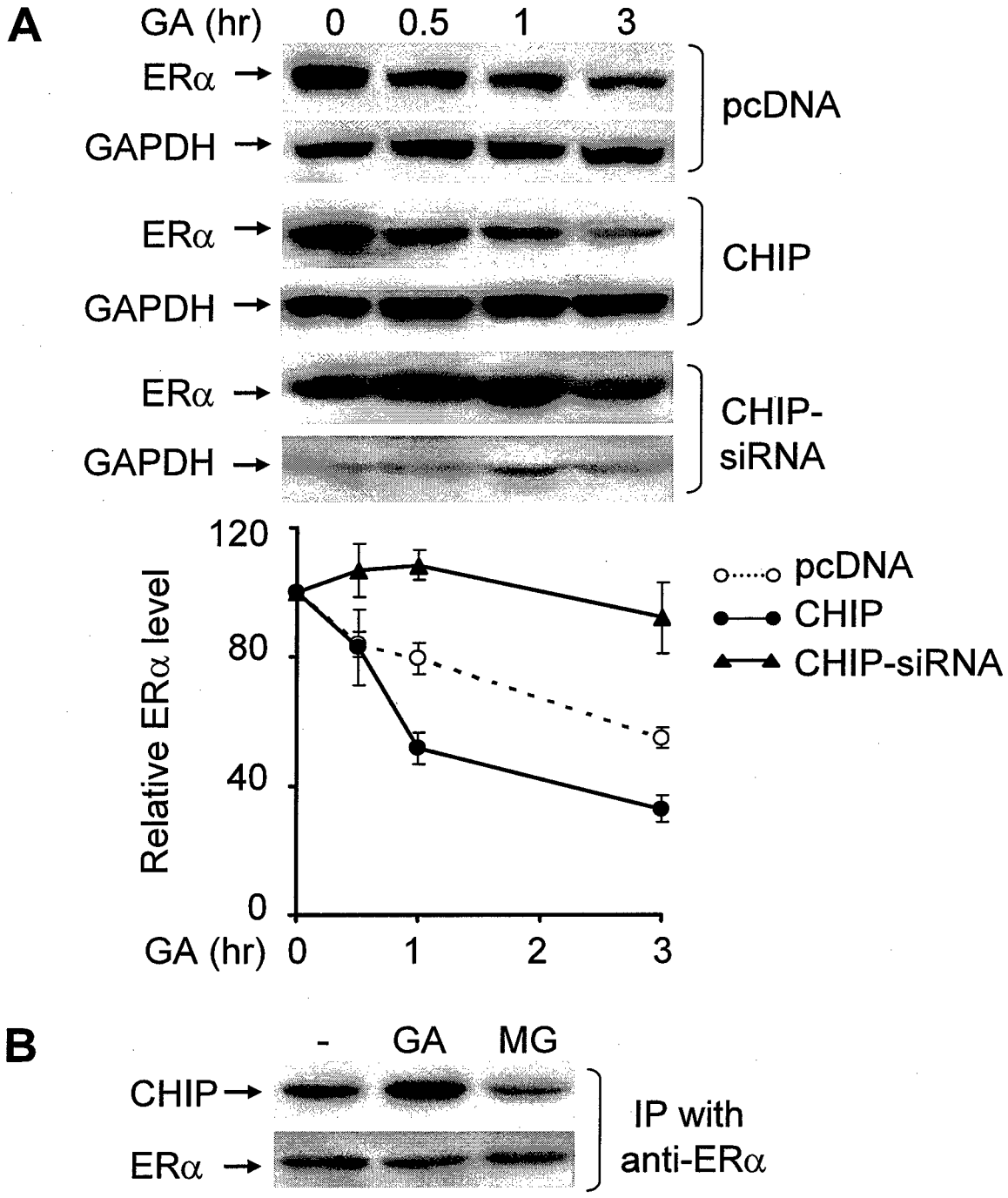
E2
*
*
pcDNA
CHIP-siRNA

Fig. 6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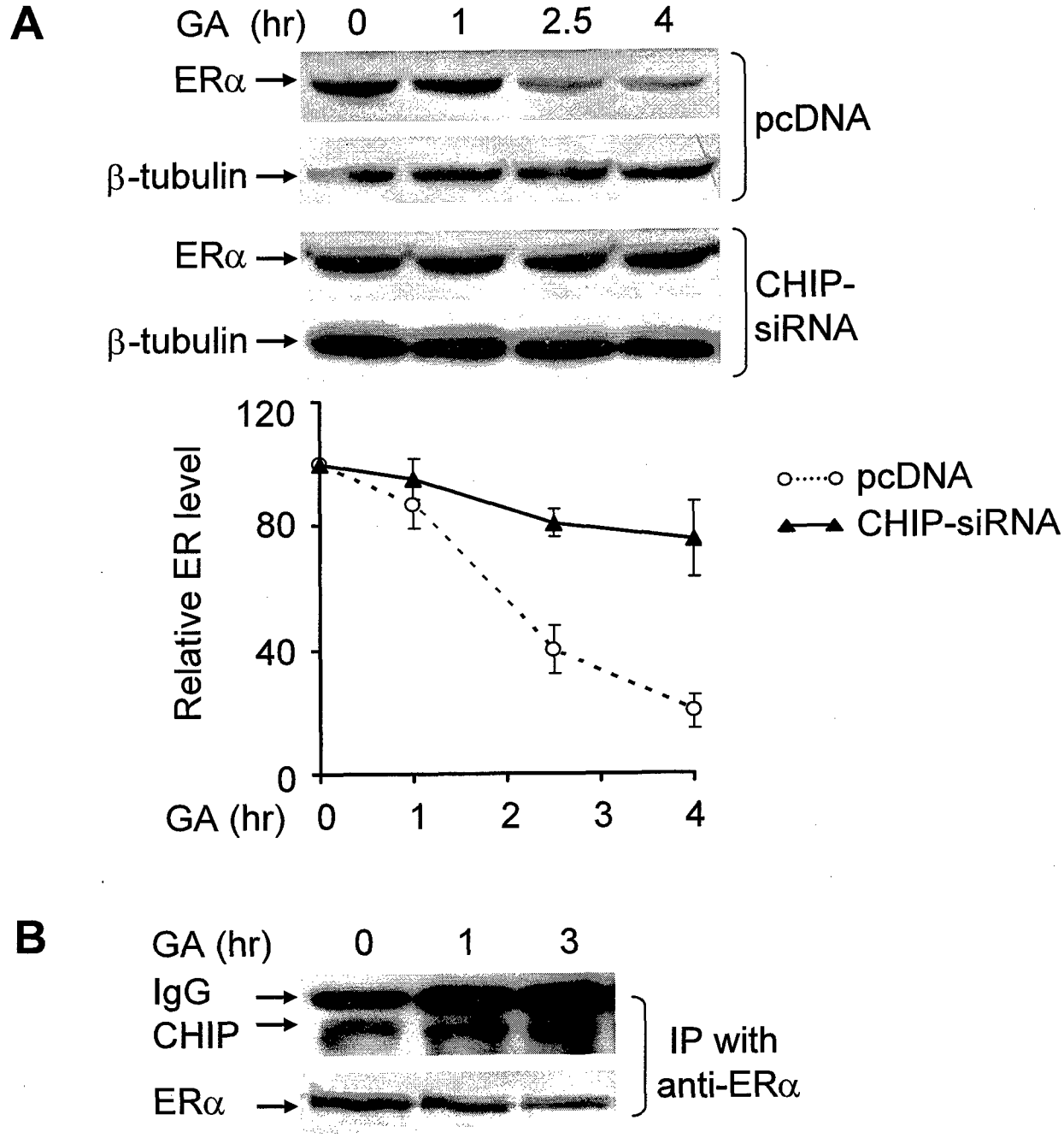
ERα
GAPDH
pcDNA
CHIP
CHIP-siRNA
Relative ERα level
120
80
40
0
GA (hr) 0 1 2 3
pcDNA
CHIP
CHIP-siRNA
B
- GA MG
CHIP
ERα
IP with anti-ERα

Fig. 7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ERα
β-tubulin
pcDNA
ERα
β-tubulin
CHIP-siRNA
120
80
40
0
Relative ER level
GA (hr) 0 1 2 3 4
pcDNA
CHIP-siRNA
B
GA (hr) 0 1 3
IgG
CHIP
ERα
IP with anti-ERα

Fig. 8

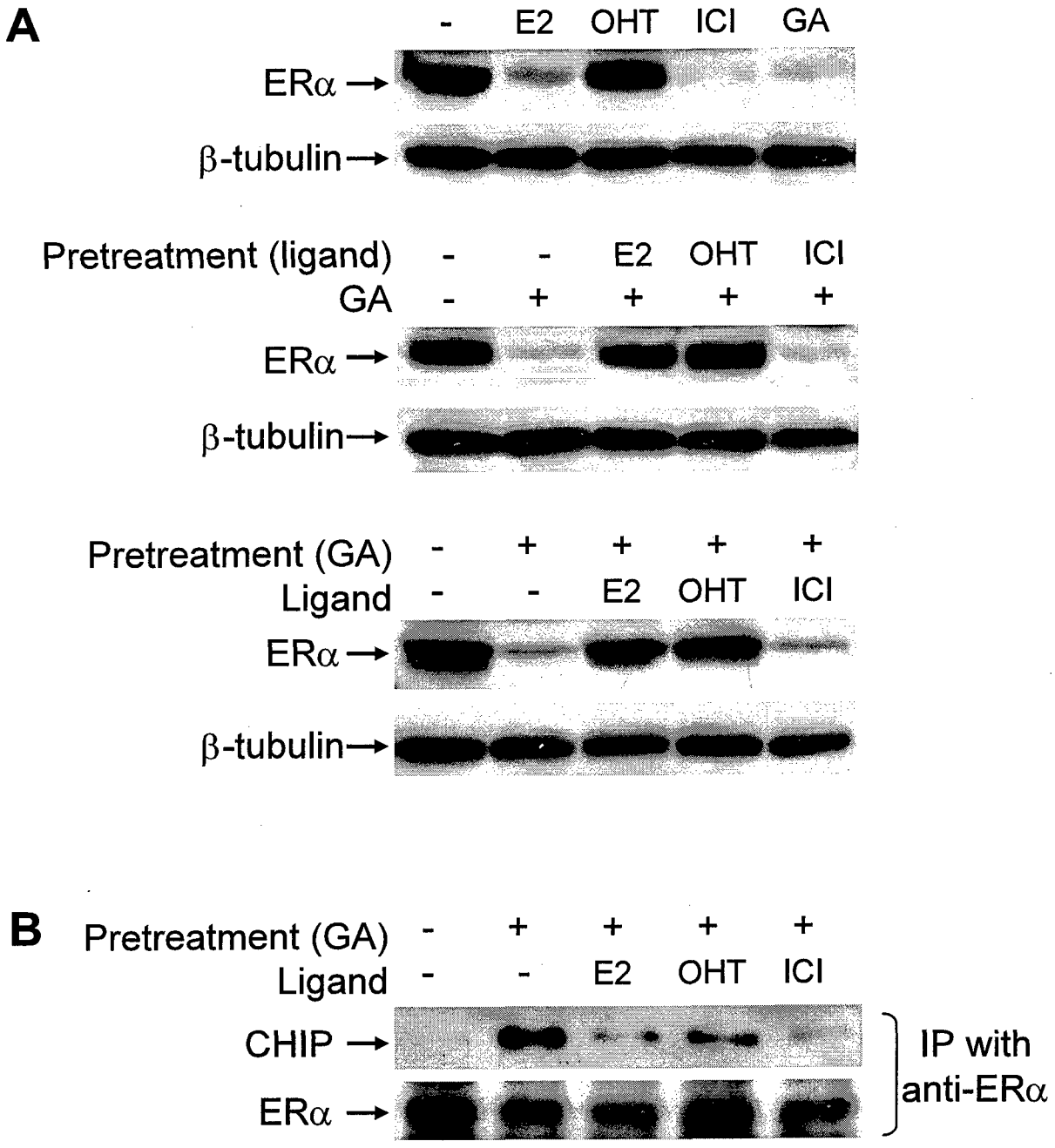
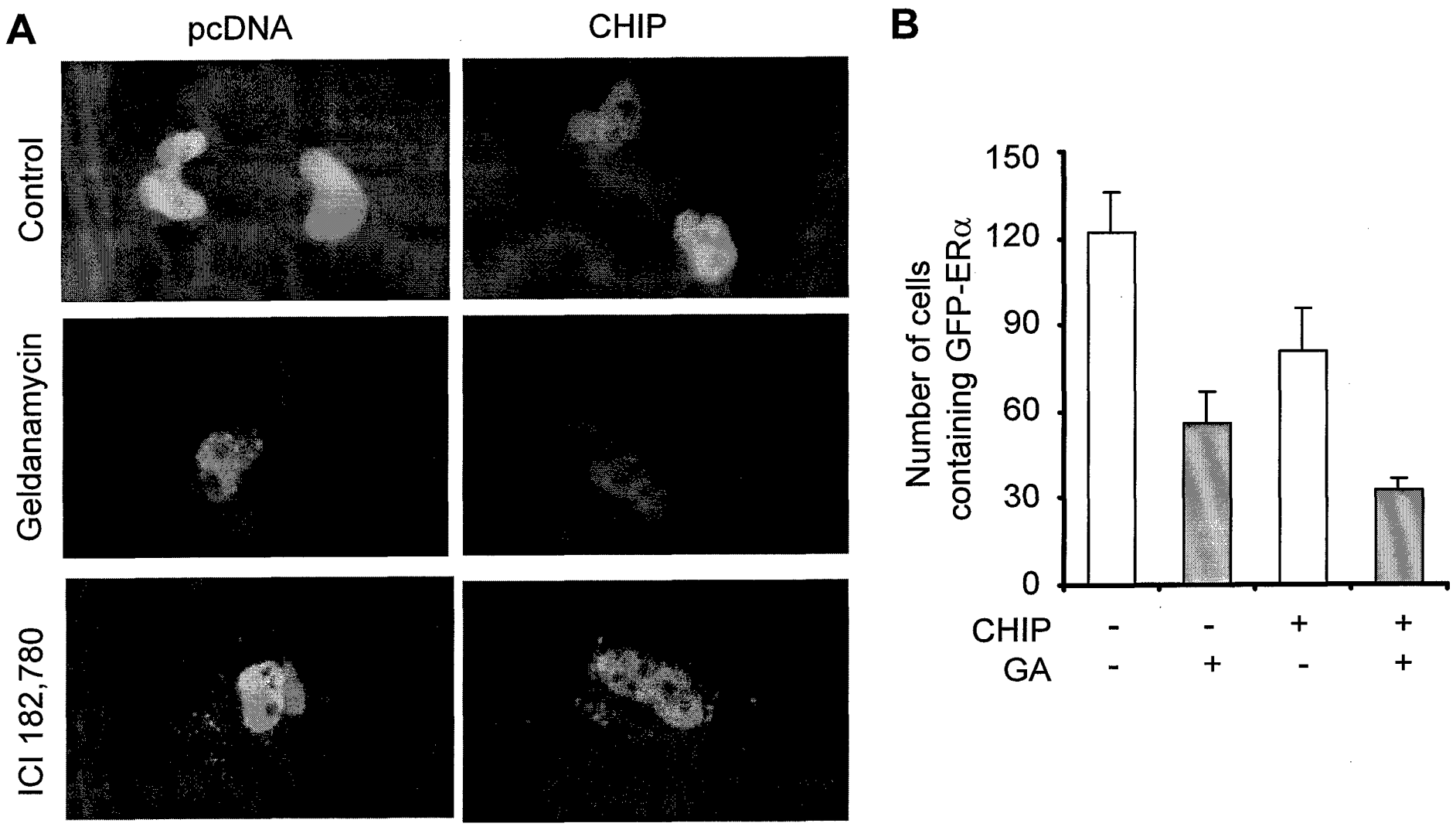

A
- E2 OHT ICI GA
ERα
β-tubulin
Pretreatment (ligand) - - E2 OHT ICI
GA - + + + +
ERα
β-tubulin
Pretreatment (GA) - + + + +
Ligand - - E2 OHT ICI
ERα
β-tubulin
B
Pretreatment (GA) - + + + +
Ligand - - E2 OHT ICI
CHIP
ERα
IP with anti-ERα

Fig. 9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CHIP
Control
Geldanamycin
ICI 182,780
B
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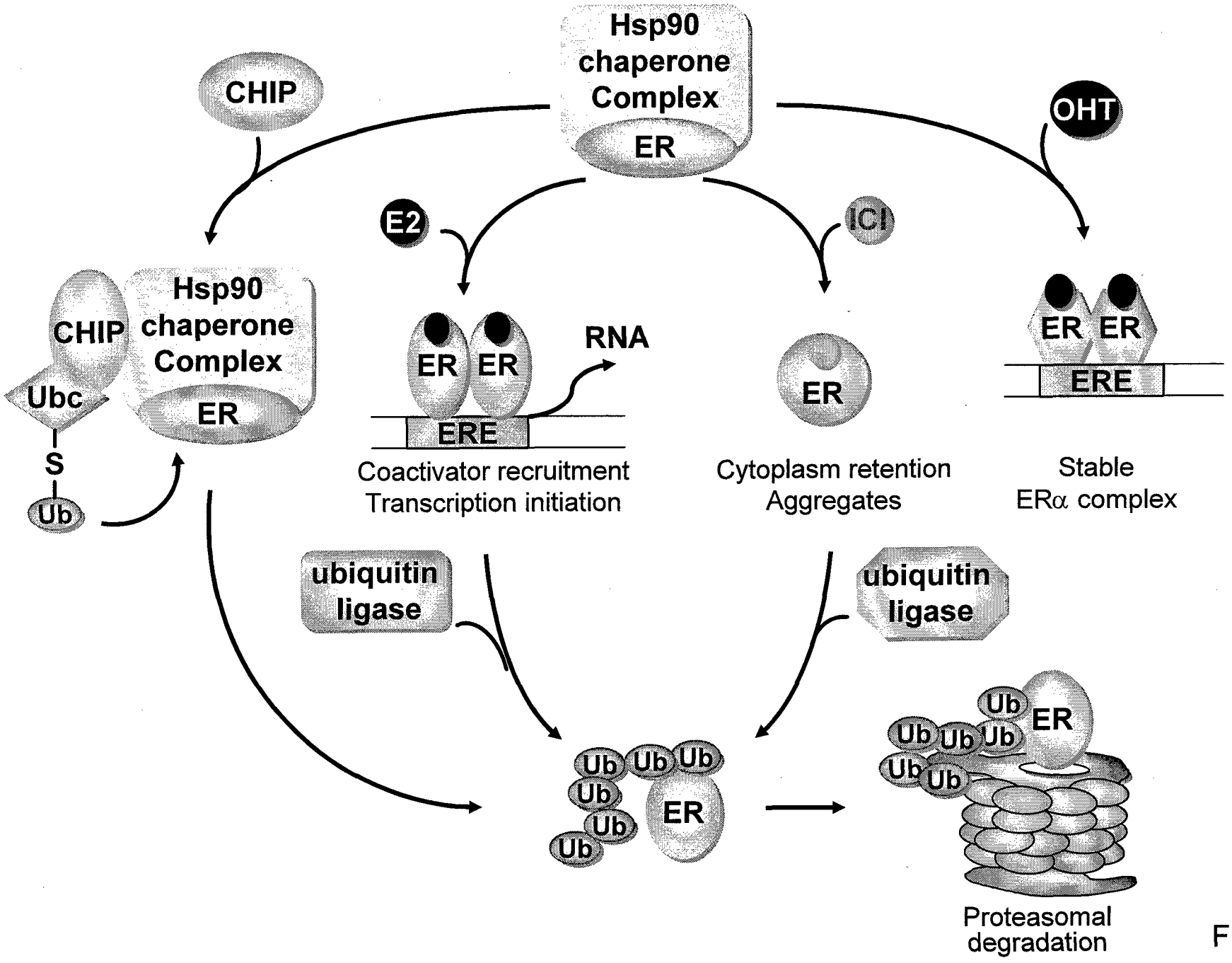
120
90
60
30
0
CHIP - - + +
GA - + - +

Fig. 10


Hsp90
chaperone
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ER
CHIP
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E2
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Ubc
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Ub
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ER ER
ERE
RNA
ER
ER ER
ERE
Coactivator recruitment
Transcription initiation
Cytoplasm retention
Aggregates
Stable
ERα complex
ubiquitin
ligase
ubiquitin
ligase
Ub
ER
Proteasomal
degradation

Fig. 11

[CANCER RESEARCH 64, 8184–8192, November 15, 2004]

Loss of Estrogen Receptor Signaling Triggers Epigenetic Silencing of Downstream Targets in Breast Cancer

Yu-Wei Leu,[1] Pearlly S. Yan,[1] Meiyun Fan,[2,3] Victor X. Jin,[1] Joseph C. Liu,[1] Edward M. Curran,[4] Wade V. Welshons,[4] Susan H. Wei,[1] Ramana V. Davuluri,[1] Christoph Plass,[1] Kenneth P. Nephew,[2,3] and Tim H-M. Huang[1]

[1]Human Cancer Genetics Program, Department of Molecular Virology, Immunology, and Medical Genetics, Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio; [2]Medical Sciences, Indiana University School of Medicine, Bloomington, Indiana; [3]Department of Cellular and Integrative Physiology and Obstetrics and Genecology, Indiana University Cancer Center, Indianapolis, Indiana; and [4]Department of Veterinary Biomedical Sciences, University of Missouri, Columbia, Missouri

ABSTRACT

Alterations in histones, chromatin-related proteins, and DNA methylation contribute to transcriptional silencing in cancer, but the sequence of these molecular events is not well understood. Here we demonstrate that on disruption of estrogen receptor (ER) α signaling by small interfering RNA, polycomb repressors and histone deacetylases are recruited to initiate stable repression of the *progesterone receptor* (*PR*) gene, a known ERα target, in breast cancer cells. The event is accompanied by acquired DNA methylation of the *PR* promoter, leaving a stable mark that can be inherited by cancer cell progeny. Reestablishing ERα signaling alone was not sufficient to reactivate the *PR* gene; reactivation of the *PR* gene also requires DNA demethylation. Methylation microarray analysis further showed that progressive DNA methylation occurs in multiple ERα targets in breast cancer genomes. The results imply, for the first time, the significance of epigenetic regulation on ERα target genes, providing new direction for research in this classical signaling pathway.

INTRODUCTION

The steroid hormone estrogen is important for normal breast development, but it is also important for growth and progression of breast cancer. The molecular actions of estrogen are mediated by estrogen receptors (ERs), ERα and ERβ. On ligand binding, ERα functions as a transcription factor by either binding to DNA targets or tethering to other transcription factors, such as AP-1 and SP-1 (1). These molecular interactions have been shown to positively or negatively modulate the activity of ERα downstream genes important to breast epithelial development.

It is known that estrogen signaling regulates the growth of some breast tumors, and antiestrogen therapies can effectively block this growth signaling, resulting in tumor suppression (2). However, most tumors eventually develop resistance to this endocrine therapy, and antiestrogens are mostly ineffective in patients with advanced disease (2). Mechanisms underlying this hormonal resistance are complex, involving intricate interactions between ERα and kinase networks (1, 2). In addition, epigenetic silencing of *ERα* is known to contribute to the antiestrogen resistance (1, 2). An emerging theme not yet investigated in this field is the subsequent influence on the expression of ERα downstream target genes.

Epigenetics can be defined as the study of heritable changes that modulate chromatin organization without altering the corresponding DNA sequence. DNA methylation, the addition of a methyl group to the fifth carbon position of a cytosine residue, occurs in CpG dinucleotides (3) and is a key epigenetic feature of the human genome. These dinucleotides are usually aggregated in stretches of 1- to 2-kb GC-rich DNA, called CpG islands, located in the promoter and first exon of ~60% of human genes (3, 4). Promoter methylation is known to participate in reorganizing chromatin structure and also plays a role in transcriptional inactivation (3, 5). Studies have suggested that the CpG island in an active promoter is usually unmethylated, with the surrounding chromatin displaying an "open" configuration, allowing for the access of transcription factors and other coactivators to initiate gene expression (6–8). Furthermore, transcription factor occupancy may make the promoter inaccessible to repressors or other chromatin-remodeling proteins. In contrast, the CpG island in an inactive promoter may become methylated, with the associated chromatin exhibiting a "closed" configuration. As a result, the methylated area is no longer accessible to transcription factors, disabling the functional activity of the promoter (7, 9, 10).

Recent studies have shown that establishing transcriptional silencing of a gene involves a close interplay between DNA methylation and histone modifications (7, 11). This process may be achieved by recruiting histone-modifying enzymes, such as histone deacetylases, which mediate posttranslational modification at the NH_2 terminus ends of histones (7, 11). As a result, chromatin modifications form distinct patterns, known as the "histone code," that may dictate gene expression (12–14).

Two models have been offered to describe the molecular sequence leading to the establishment of epigenetic gene silencing. One model suggests that histone modifications are the primary initiating event in transient repression (15, 16). DNA methylation subsequently accumulates in the targeted CpG island, creating a heterochromatin environment to establish a heritable, long-term state of transcriptional silencing. However, a second model is that DNA methylation can actually specify unique histone codes for maintaining the silenced state of a gene (17–20). In this case, DNA methylation may precede histone modifications. Clearly, this epigenetic process is complex, and multiple systems may be implemented for genes participating in different signaling pathways.

In this study, we investigated whether the removal of ERα signaling triggers changes in DNA methylation and chromatin structure of ERα target promoters. By using RNA interference (RNAi) to transiently disable ERα in breast cancer cells, we show, for the first time, that polycomb repressors and histone deacetylases assemble on the promoters of interrogated ERα target genes to participate in long-term transcriptional silencing. These events are later accompanied by a progressive accumulation of DNA methylation in the promoter re-

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Note: Supplementary data for this article can be found at Cancer Research Online (http://cancerres.aacrjournals.org). T. Huang is a consultant to Epigenomics, Inc., Berlin, Germany.

Requests for reprints: Tim H-M. Huang, Human Cancer Genetics Program, Department of Molecular Virology, Immunology, and Medical Genetics, Comprehensive Cancer Center, The Ohio State University, 420 West 12th Avenue, Columbus, OH 43210. Phone: 614-688-8277; Fax: 614-292-5995; E-mail: huang-10@medctr.osu.edu.

gions of the now silent targets, leaving a heritable "mark" that may be stably transmitted to cell progeny.

MATERIALS AND METHODS

Cell Lines and Clinical Samples. The breast cancer cell line MCF-7 and its derived subline, C4-12, were routinely maintained in our laboratories. For the demethylating treatment, cells were plated at a density of 2×10^6 cells per 10-cm dish and pretreated with 2 or 5 μmol/L 5-aza-2′-deoxycytidine (5-AzadC; Sigma, St. Louis, MO) for 5 days before treatment with 17β-estradiol (E_2; 10 nmol/L, 24 hours). Thirty-two invasive ductal carcinomas were obtained from patients undergoing breast surgery at the Ellis Fischel Cancer Center (Columbia, MO), in compliance with the institutional review board. Seven tumor-free breast parenchymas were used as controls. The ER status of tumor tissue was determined by immunohistochemical staining (21).

Transfection of Estrogen Receptor α Small Interfering RNAs. MCF-7 cells (60% confluent in a 3.5-cm–diameter culture dish) were starved in serum-free medium (minimal essential medium only) for 72 hours, followed by the addition of 10 nmol/L E_2 (E2758; Sigma) for 24 hours. The cells were then transfected with small interfering RNAs (siRNAs) for 4 to 5 hours with DMRIE-C reagent (Invitrogen, Carlsbad, CA). Double-stranded siRNA was generated using the *Silencer* siRNA Construction Kit (Ambion, Austin, TX). The siRNA oligonucleotides designed according to the ERα mRNA sequence (GenBank accession numbers AF_258449, 258450, and 258451) are as follows: (*a*) target sequence 1 (5′-AACCTCGGGCTGTGCTCTTTT), sense strand siRNA primer 5′-CCTCGGGCTGTGCTCTTTTTTCCTGTCTC and antisense strand siRNA primer AAAAGAGCACAGCCCGAGGTTCCTG-TCTC; and (*b*) target sequence 17 (5′-AAACAGGAGGAAGAGCTGCCA), sense strand siRNA primer 5′-ACAGGAGGAAGAGCTGCCATTCCT-GTCTC and antisense strand siRNA primer 5′-TGGCAGCTCTTCCTCCT-GTTTCCTGTCTC.

Media were changed after transfection. The cells were then harvested for total RNA (RNeasy Kit; Qiagen, Valencia, CA) and genomic DNA (QIAamp; Qiagen) isolation at various time periods after siRNA treatment.

Transfection of Estrogen Receptor α Expression Vector. C4-12 cells were transfected with pcDNA-ERα (C4-12/ER) or empty vector (C4-12/vec) using LipofectAMINE Plus Reagent (Life Technologies, Inc., Carlsbad, CA) and then exposed to an antibiotic (G418; 0.5 mg/mL) for 3 weeks. Expression of ERα in G418-resistant colonies was detected by immunoblotting with an anti-ER antibody (Chemicon, Temecula, CA).

Real-Time Reverse Transcription-Polymerase Chain Reaction. Total RNA (2 μg) was treated with DNase I to remove potential DNA contamination and then reverse transcribed using the SuperScript II reverse transcriptase (Invitrogen). Real-time polymerase chain reactions (PCRs) were then performed using puReTaq Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ) and monitored by SYBR Green I (BioWhittaker, Walkersville, MD) using a Smart Cycler Real-Time PCR instrument (Cepheid, Sunnyvale, CA) for 42 cycles. PCR products of the expected size were also visualized on agarose gels stained with ethidium bromide. Alternatively, the reverse transcription-PCR (RT-PCR) reaction was conducted using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) in an iCycler system (Bio-Rad) for *PR* transcripts (22). The relative mRNA level of a given locus was calculated by Relative Quantitation of Gene Expression (Applied Biosystems, Foster City, CA) with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) or *β-actin* mRNA as an internal control. The primers used for RT-PCR reactions are listed in Supplementary Table S1.

Immunofluorescence and Western Blot Analysis. MCF-7 cells (2×10^5) treated with or without ERα siRNAs were permeablized with 0.5% Nonidet P-40/PBS and blocked with a 1:100 dilution of horse serum before incubation with primary anti-ERα antibody (1:1,000; mouse monoclonal antibody D-12; Santa Cruz Biotechnology, Santa Cruz, CA). Sample slides were washed with PBS and incubated in the dark with secondary antibody (1:500) conjugated with Texas Red (fluorescent antimouse IgG kit; Vector Laboratories, Burlingame, CA) for 1 hour. The slides were then mounted with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories) and observed under a fluorescence microscope (Zeiss Axioskop 40; Zeiss, Thornwood, NY). Images were captured by the AxioCam HRC camera and analyzed by AxioVision 5.05 software.

Small interfering RNA-treated cells and control cells were lysed in the presence of proteinase inhibitors. One hundred micrograms of protein were subjected to 7% SDS-PAGE and transferred to immunoblot membranes. The membranes were then incubated with mouse anti-ERα (MAB463; Chemicon) and labeled secondary antibody. GAPDH was used a loading control.

Chromatin Immunoprecipitation-Polymerase Chain Reaction. Cultured cells (2×10^6) were cross-linked with 1% formaldehyde and then washed with PBS in the presence of protease inhibitors. The cells were resuspended in lysis buffer, homogenized using a tissue grind pestle to release nuclei, and then pelleted by centrifugation. SDS-lysis buffer from a chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology, Lake Placid, NY) was used to resuspend the nuclei. The lysate was sonicated to shear chromatin DNAs and then centrifuged to remove cell debris. The supernatants were transferred to new tubes and incubated overnight with an antibody against ERα, YY-1, or EZH2 (Santa Cruz Biotechnology); HDAC1, MBD2, or MeCP2 (Upstate Biotechnology); and DMNT1, DNMT3a, or DNMT3b (Imgenex, San Diego, CA). Agarose slurry was then added to the mixture, and the chromatin-bound agarose was centrifuged. The supernatant was collected and used for total input (it serves as a positive control) in the ChIP-PCR assay. After elution, proteins were digested from the bound DNA with proteinase K. Phenol/chloroform-purified DNA was then precipitated and used in ChIP-PCR assays for a *progesterone receptor* (*PR*) promoter region. The primer sequences were 5′-GGCTTTGGGCGGGGCCTCCCTA (sense strand) and 5′-TCTGCTGGCTCCGTACTGCGG (antisense strand). After amplification, ^{32}P-incorporated PCR products were separated on 8% polyacrylamide gels and subjected to autoradiography using a Storm PhosphorImager (Amersham Biosciences).

Methylation-Specific Polymerase Chain Reaction. Genomic DNA (1 μg) from each sample was bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research Corp., Orange, CA), according to the manufacturer's protocol. The converted DNA was eluted with 40 μL of elution buffer and then diluted 50 times for methylation-specific PCR (MSP). The primer sets designed for amplifying the methylated or unmethylated allele of the PR locus are listed in Supplementary Table S2. All PCR reactions were performed in PTC-100 thermocyclers (MJ Research, Watertown, MA) using AmpliTaq Gold DNA polymerase (Applied Biosystems). ^{32}P-incorporated amplified products were separated on 8% polyacrylamide gels and subjected to autoradiography using a Storm PhosphorImager (Amersham Biosciences).

Combined Bisulfite Restriction Analysis. Combined bisulfite restriction analysis (COBRA) was carried out essentially as described previously (23). Bisulfite-modified DNA (~10 ng) was used as a template for PCR with specific primers flanking the interrogated sites (*Taq*I or *Bst*UI) of an ERα downstream target. Primer sequences used for amplification are listed in Supplementary Table S3. After amplification, radiolabeled PCR products were digested with *Taq*I or *Bst*UI, which restrict unconverted DNA containing methylated sites. The undigested control and digested DNA samples were run in parallel on polyacrylamide gels and subjected to autoradiography. The percentage of methylation was determined as the intensity of methylated fragments relative to the combined intensity of unmethylated and methylated fragments.

Chromatin Immunoprecipitation on Chip. MCF-7 cells (2×10^7) were used to conduct ChIP with an antibody specific for ERα following the protocol described (see Chromatin Immunoprecipitation-Polymerase Chain Reaction). After chromatin coimmunoprecipitation, DNA was labeled with Cy5 fluorescence dye and hybridized to a genomic microarray panel containing ~9,000 CpG islands (24). Microarray hybridization and posthybridization washes have been described previously (25). The washed slides were scanned by a Gene Pix 4000A scanner (Axon, Union City, CA), and the acquired microarray images were analyzed with GenePix Pro 4.0 software. This ChIP-on-chip experiment was conducted twice.

Positive CpG island clones were sequenced, and the derived sequences were used to identify putative transcription start sites by Blastn[5] or Blat.[6] Both Genomatrix[7] and TFSEARCH[8] programs were then used to localize the consensus sequences of the estrogen response elements (EREs) and other

[5] http://www.ncbi.nlm.nih.gov/BLAST/.
[6] http://genome.cse.ucsc.edu/cgi-bin/hgBlat.
[7] http://www.genomatix.de/site_map/index.html.
[8] http://www.cbrc.jp/research/db/TFSEARCH.html.

related transcription factor binding sites (AP-1, SP-1, cAMP-responsive element binding protein, and CEBP).

Differential Methylation Hybridization. Differential methylation hybridization (DMH) was performed essentially as described previously (25, 26). Briefly, 2 μg of genomic DNA were digested by the 4-base frequent cutter *Mse*I, which restricts bulk DNA into small fragments but retains GC-rich CpG island fragments (24). H-24/H-12 PCR linkers (5′-AGGCAACTGTGCTATC-CGAGGGAT-3′ and 5′-TAATCCCTCGGA-3′) were then ligated to the digested DNA fragments. The DNA samples were further digested with two methylation-sensitive endonucleases, *Hpa*II and *Bst*UI, and amplified by PCR reaction using H-24 as a primer. After amplification, test DNA from siRNA-treated cell lines or clinical samples was labeled with Cy5 (red) dye, whereas control DNA from the mock-transfected cell lines or normal female blood samples was coupled with Cy3 (green) dye. Equal amounts of test and control DNAs were cohybridized to a microarray slide containing 70 ERα promoter targets (average, 500 bp) identified from the ChIP-on-chip results. Posthybridization washing and slide scanning are described above. Normalized Cy5/Cy3 ratios of these loci were calculated by GenePix Pro 4.0.

Shrunken Centroids Analysis. DMH microarray data were analyzed by the procedure described online.[9] This program incorporates graphic methods for automatic threshold choice and centroid classification.

Statistical Analyses. Differences of methylation or mRNA levels in experimental studies were analyzed by a paired *t* test. Methylation differences between two tumor groups were determined with a Pearson's χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS

RNA Interference Transiently Knocks Down Estrogen Receptor α Expression in Breast Cancer Cells. Although several *in vitro* systems and mouse models are available for analysis of estrogen signaling, to our knowledge, the recently described RNAi (27) has not been actively used in this area of research. We therefore used this technology to specifically repress *ERα* gene expression via targeted RNA degradation (28, 29). Six different *ERα* siRNAs, two of which have sequences homologous to a splice variant, were synthesized (Fig. 1*A*). These siRNAs (40 nmol/L) were individually transfected into MCF-7, an ERα-positive human breast cancer cell line. MCF-7 cells were cultured in the presence of E_2. Quantitative RT-PCR analysis showed that, 24 hours after transfection, two siRNAs, siRNAs 1 and 17, were capable of repressing ERα transcripts (Fig. 1*B*). Specifically for siRNA 1, we observed a >93% decrease of *ERα* mRNA. Immunofluorescence (Fig. 1*C*) and Western blot (Fig. 1*D*) analyses confirmed that this RNAi also dramatically reduced ERα protein synthesis. This inhibitory effect appeared to be transient, and the expression of ERα protein reappeared in cultured cells 4 weeks after RNAi withdrawal (Fig. 1*D*).

Epigenetic Silencing of the *PR* Gene Is Triggered by Estrogen Signal Disruption. We hypothesized that disruption of ERα signaling by siRNA may lead to the silencing of some positively regulated ERα targets governed by epigenetic mechanisms. To this end, a known ERα downstream target, the *PR* gene, was investigated in detail. In Fig. 2*A* and *B*, quantitative RT-PCR analysis showed that by 36 hours after treatment of MCF-7 cells with siRNA 1, the level of *PR* transcripts (PR-A and PR-B) was reduced by >95% (paired *t* test, $P < 0.0001$). Next, ChIP-PCR was performed to determine the status of chromatin remodeling at the 5′-end of the *PR* gene. The protein-DNA complexes were immunoprecipitated with antibodies to ERα or to specific modified histones (acetyl-H3, acetyl-H3-K9, and methyl-H3-K4) known to specify active transcription (7, 30). As shown in Fig. 2*C*, the presence of these active chromatin components was diminished over a period of 36 hours, coinciding with decreased ERα binding to the *PR* promoter region.

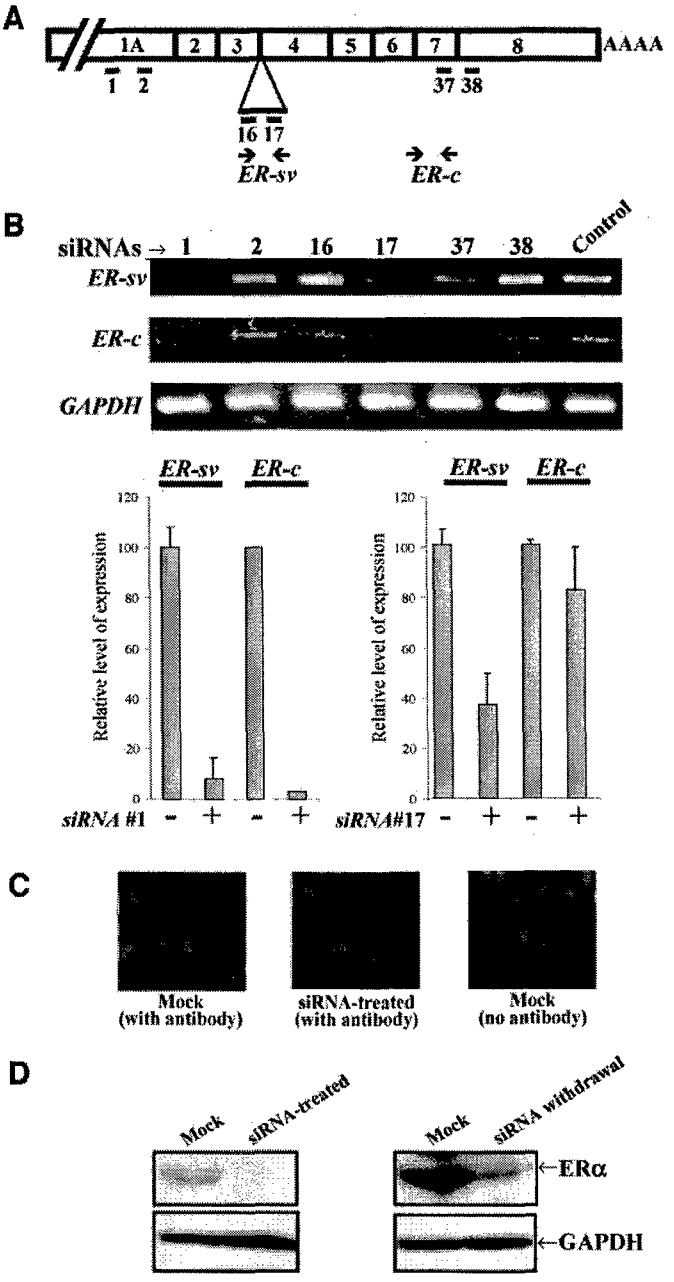

Fig. 1. Down-regulation of *ERα* by siRNAs. MCF-7 ERα-positive human breast cancer cells were transfected with six different double-stranded siRNAs for 4 hours. Cells were left untreated for an additional 24 hours, and total RNA and proteins were then harvested for analysis. Additionally, cells were left untreated for a prolonged period of 3 to 4 weeks (see RNAi withdrawal in *D*), and proteins were harvested for analysis. Mock-treated cells were transfected with vehicle only. *A*, map of *ERα* cDNA. The *ERα* mRNAs were transcribed from several unique first exons and seven other common exons. An additional splice variant (between exons 3 and 4) is also shown. Approximate locations of siRNAs (*1*, *2*, *16*, *17*, *37*, and *38*) are indicated by *bars*. *B*, RT-PCR of *ERα*. PCR primers specifically for the splice variant (*ER-sv*) and the common transcripts (*ER-c*) are indicated by *arrows* in *A*. The *GAPDH* transcript was used as a control. The expression levels of *ER-sv* or *ER-c* were normalized against *GAPDH* (*bottom panel*) by real-time PCR. *C*, immunofluorescence analysis of ERα expression. Small interfering RNA 1- or mock-treated MCF-7 cells were cultured on microscope coverslips and incubated with an ERα antibody. Positive nuclei staining (Texas Red) was seen only in mock-treated cells (with antibody), but not in siRNA 1-treated (with antibody) or mock-treated (without antibody) cells. 4′,6-Diamidino-2-phenylindole was used to counterstain nuclei (*blue*). *D*, Western blot analysis of ERα expression. Cellular protein products from various treatments were harvested and separated by SDS-PAGE for immunoblot analysis with ERα and GAPDH (loading control) antibodies, respectively. Results are representative of at least three independent experiments.

[9] http://www-stat.stanford.edu/~tibs/PAM.

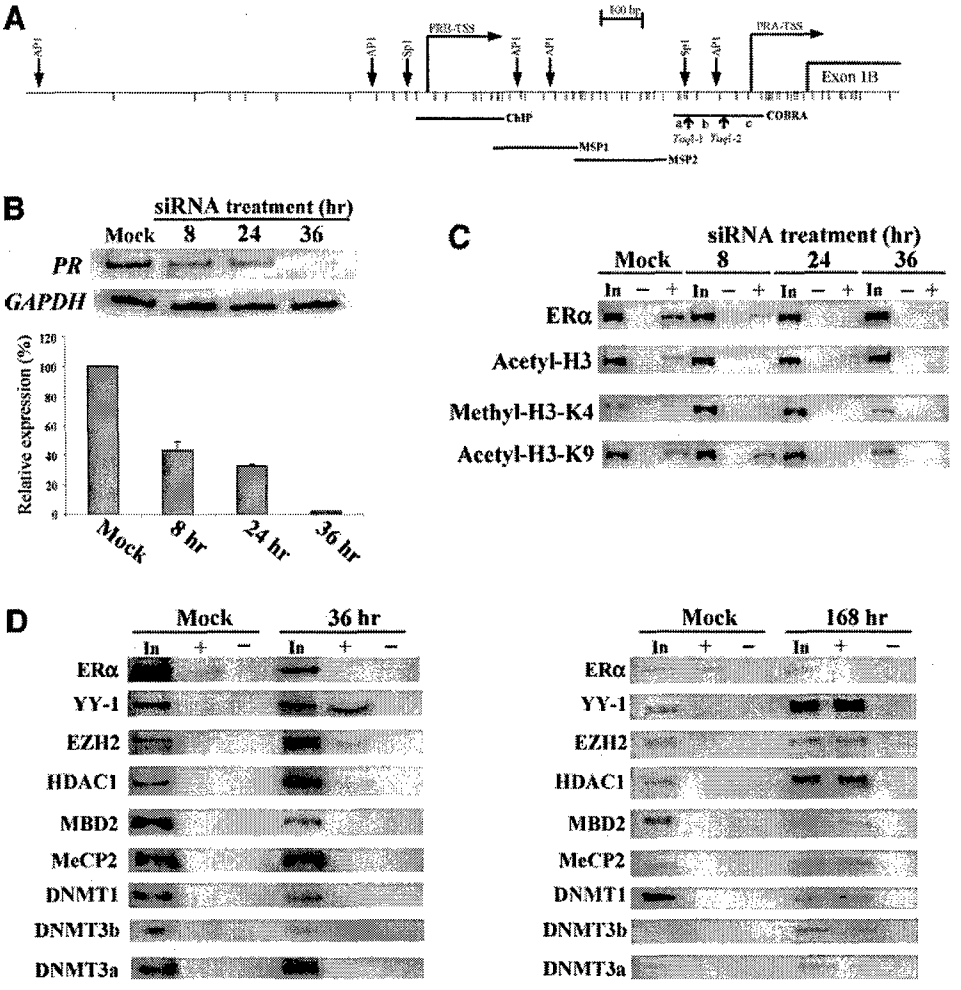

Fig. 2. Loss of estrogen signaling leads to epigenetic silencing of the *PR* gene. *A*, genomic map of the *PR* promoter CpG island. The positions of CpG sites in the genomic sequence are indicated by *thin vertical lines*. The positions of two alternative transcription start sites, *PRA* and *PRB*, respectively, are indicated by *bent arrows*. Potential transcription factor binding sites (AP-1 and Sp1) resulting from a TFSEARCH query (www.cbrc.jp/htbin/nph-tfsearch) are marked by *vertical arrows*. The location of the *PR* promoter fragment used for the ChIP assay and the two regions (MSP1 and MSP2) used for MSP are indicated by *horizontal lines* (see Fig. 3*A*). The region amplified for COBRA is *underlined*, and the *two vertical arrows* indicate the interrogating *Taq*I restriction (TCGA) sites (see Fig. 3*C*). *B*, time course inhibition of PR transcripts by siRNA treatment. MCF-7 cells were transfected with ERα siRNA 1 for the indicated time periods (8, 24, and 36 hours) and harvested for real-time RT-PCR. Mock-transfected cells were harvested at the 36 hour time point. Primers were designed to amplify a common region of the two known *PR* transcripts. Relative levels of *PR* transcripts were normalized against that of the *GAPDH* loading control. Results from three independent experiments are shown as means ± SE. *C*, ChIP-PCR assay for activating chromatin modifications on the PR CpG promoter island. Chromatin DNA was immunoprecipitated with antibodies specific for ERα, acetylated histone H3 (acetyl-H3 and/or acetyl-H3-K9), or dimethyl-H3-K4. DNA fragments were amplified with a primer pair located in a *PR* CpG island region (indicated in *A*). The final radiolabeled products were separated on 6% polyacrylamide gels and subjected to autoradiography. *In*, total input; −, without antibody; +, with antibody. *D*, ChIP-PCR assays for repressive chromatin modifications on the PR promoter CpG island region at 36 and 168 hours after ERα siRNA treatment. Antibodies against polycomb repressors (YY-1 and EZH2), histone deacetylase (HDAC1), methyl-CpG binding proteins (MBD2 and MeCP2), and DNA methyltransferases (DMNT1, DNMT3a, and DNMT3b) were used in ChIP-PCR assays. *In*, total input; −, without antibody; +, with antibody.

We speculated that this initial transcriptional inactivation might trigger further recruitment of repressor molecules to the *PR* promoter CpG island to subsequently establish a long-term silencing state. ChIP-PCR assays were conducted with a panel of antibodies raised for the polycomb repressors YY-1 and EZH2, histone deacetylase HDAC1, methyl-CpG–binding proteins MBD2 and MeCP2, and DNA methyltransferases DMNT1, DNMT3a, and DNMT3b. At 36 hours after siRNA treatment, YY-1 and EZH2 were bound to the promoter region (Fig. 2*D*). These polycomb proteins have previously been shown to target the regulatory regions of *homeobox* genes, the resulting repression of which can be tissue specific and important for early embryonic development (31, 32). Here we demonstrate for the first time that these proteins have an additional role in repressing an ERα target gene. Furthermore, the *PR* promoter was seen, at 36 hours, to recruit HDAC1 (Fig. 2*D*), a protein known to deacetylate histone protein tails, creating a repressive heterochromatin environment in the targeted promoter area (7, 9, 10). However, at this early time point (36 hours), ChIP-PCR analysis did not detect the presence of the DNA methyltransferase DNMT1 in the *PR* promoter CpG island area (Fig. 2*D*), nor did we observe the presence of MBD2 or MeCP2, which are known to bind methylated CpG sites. Only a faint band corresponding to DNMT3b was detected in the *PR* promoter area by 36 hours after ERα siRNA treatment of MCF-7 cells. Except for DNMT3a, the recruitment of these repressive proteins to the *PR* promoter CpG island was evident by 168 hours after siRNA treatment.

To further determine whether the recruitment of these epigenetic components could trigger *de novo* DNA methylation, MSP assays (33) were conducted to survey two 5′-end regions of *PR* at different time periods after siRNA treatment. As shown in Fig. 3*A*, *PR* methylation was detected in amplified bisulfite-treated DNA only at 36 hours after treatment. This observation was independently confirmed by conducting semiquantitative COBRA (23). In the assay, ~10% of MCF-7 cells showed methylation in one (Fig. 2*A*, *TaqI-2*) of the two PR *Taq*I sites analyzed 36 hours after siRNA treatment (Fig. 3*B*). Both of these sites became methylated at a later time point (168 hours) of treatment (Fig. 3*B*). This study implies that acquired DNA methylation is a late event and that the density of DNA methylation may gradually accumulate at the 5′-end of *PR* after disrupting ERα signaling by siRNA.

Next, we determined whether this *acquired* promoter methylation could be observed in ERα-negative breast tumors. COBRA was therefore conducted in 32 primary tumors (16 ERα-negative and 16 ERα-positive tumors) and 7 normal controls (see representative examples in Fig. 3*C*). Consistent with the *in vitro* findings, *PR* promoter hypermethylation occurred more frequently in ERα-negative tumors (45%) than in ERα-positive tumors (10%) (χ^2 test, $P < 0.05$).

Reexpression of *PR* Requires Both Estrogen Signal Restoration and DNA Demethylation. The *in vitro* experimental results described above are based on transient siRNA treatment. To determine whether this signal disruption has a lasting impact on *PR* expression, we took advantage of an ERα-negative cell subline, C4-12, derived from ERα-positive MCF-7 cells by long-term hormonal depletion (34). A recent study has indicated that *PR* gene expression is absent in this cell line (35). We therefore examined whether stably reexpressing ERα could restore *PR* gene activity in several established C4-12

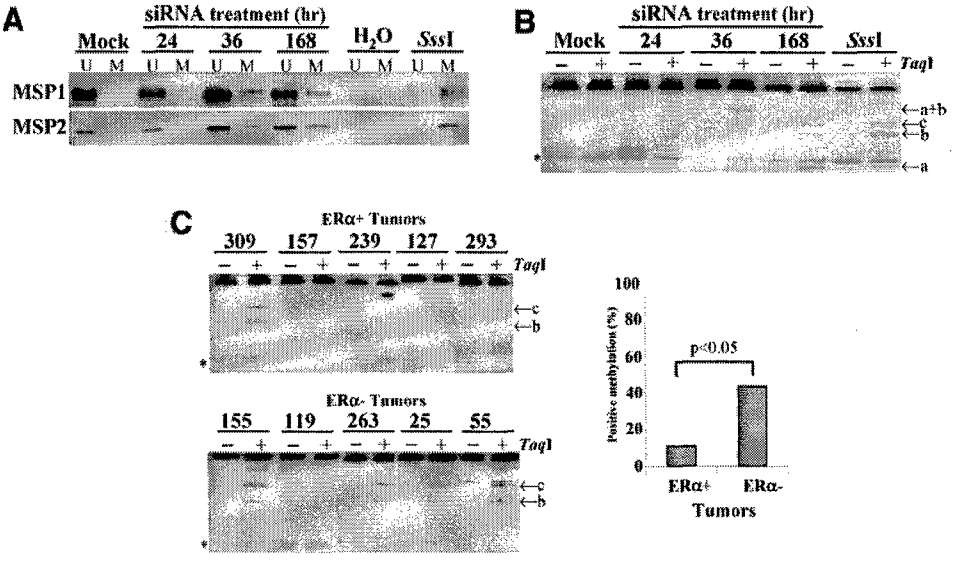

Fig. 3. DNA methylation analysis of the *PR* promoter by MSP and COBRA. *A*, bisulfite-treated DNA samples from siRNA- and mock-treated cells were used for amplification with specific primers for MSP1 and MSP2 (see Fig. 2*A*). Radiolabeled PCR products for unmethylated (*Lanes U*) and methylated (*Lanes M*) DNA strands were separated on 6% polyacrylamide gels. *B*. For COBRA, bisulfite DNA samples from siRNA- and mock-treated cells were amplified and digested with *Taq*I enzyme and then separated on polyacrylamide gels. The digested DNA fragments (*a*, *b*, and *c*) indicated by the *arrows* reflect methylation of *Taq*I restriction sites within the *PR* promoter CpG island (see Fig. 2*A*). An *asterisk* indicates PCR artifact or primer-dimer. *C*, COBRA of *PR* promoter in ERα-positive and -negative breast tumors. The percentage of positive methylation was calculated as the intensity of methylated fragments relative to the combined intensity of unmethylated and methylated fragments (*right panel*). All DNA methylation data shown here are representative of at least three independent experiments.

subclones (C4-12/vec, C4-12/ER#1, C4-12/ER#50, and C4-12/ER#86; see examples in Fig. 4*A*, *inset*).

Treatment of these subclones (*e.g.*, C4-12/ER#86 in Fig. 4*A*) with E_2, however, failed to induce *PR* mRNA expression, demonstrating that reintroduction of ERα alone was insufficient to reactivate expression of a silent *PR* gene. To determine whether loss of *PR* expression was due to DNA methylation, C4-12/vec (*i.e.*, cells stably transfected with empty vector) and C4-12/ER#86 cells were pretreated with 5-AzadC, a DNA demethylating agent, before E_2 treatment. As shown in Fig. 4*A*, sequential treatment with 5-AzadC followed by E_2 resulted in reexpression of *PR* mRNA in C4-12/ER#86 cells, but not in C4-12/vec cells, demonstrating that both ERα expression and DNA demethylation are required to restore PR expression. To further confirm that reactivation of the *PR* gene was due to DNA demethylation, the methylation status of the *PR* promoter CpG island region was examined by MSP (Fig. 4*B*). In contrast to MCF-7 cells in which the *PR* promoter CpG island was unmethylated (Fig. 3*A*), methylation was observed in both C4-12/vec and C4-12/ER cells (Fig. 4*B*, *Lanes 1*, 2, and *5–10*). However, after treatment with 5-AzadC, *PR* promoter methylation was partially reversed in C4-12/vec cells (Fig. 4*B*, *Lanes 3* and *4*) and completely removed in C4-12/ER#86 cells (Fig. 4*B*, *Lanes 11* and *12*). Together, these results demonstrate that the silencing of *PR* is maintained, in part, by DNA methylation in the ERα-negative C4-12 cells and that reactivation of the *PR* promoter requires both the presence of ERα and DNA demethylation.

DNA Methylation of Multiple Estrogen Receptor α Downstream Targets Is Triggered by Disrupting Receptor Signaling. To determine whether this epigenetically mediated silencing is a generalized event, we used ChIP-on-chip, a novel microarray-based method developed in our laboratory (36, 37), for a genome-wide screening of ERα downstream targets. In this case, we probed a panel of ~9,000 arrayed CpG island fragments with anti-ERα–coimmuo-

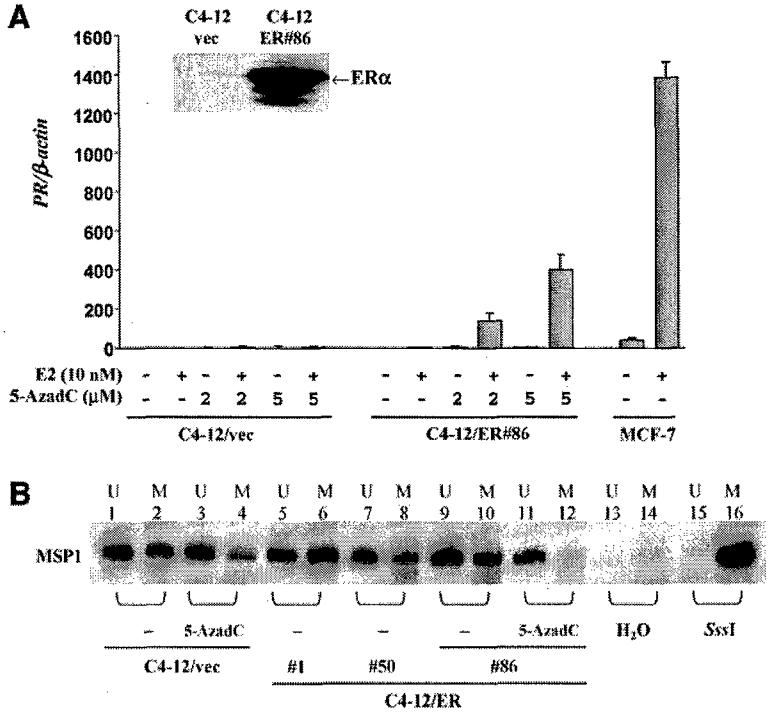

Fig. 4. Treatment of C4-12/ER cells with 5-AzadC restores *PR* mRNA expression. *A*. Expression levels of *PR* mRNA in C4-12/Vec and C4-12/ER cells were determined after treatment with the indicated doses of 5-AzadC for 5 days, followed by 10 nmol/L E_2 for 24 hours. *PR* mRNA levels were measured by quantitative real-time RT-PCR and normalized using *β-actin* mRNA levels. The results ($X \pm$ SE) are shown from two independent experiments, each in triplicate. ERα expression in C4-12 cells #86 (*A*, *inset*) was detected by immunoblotting with an anti-ERα antibody. *B*, analysis of *PR* promoter by MSP assay. Bisulfite-treated DNAs from cells treated with or without 5-AzadC were amplified with specific primers for the *PR* promoter CpG island (*i.e.*, the MSP1 region in Fig. 2*A*). Radiolabeled PCR products for unmethylated (*Lanes U*) and methylated (*Lanes M*) DNA strands were separated on 6% polyacrylamide gels. MSP data shown here are representative of three independent experiments.

precipitated chromatins. Putative target sequences were used to search for the presence of ERα binding motifs, EREs, and other related binding sites (*e.g.*, AP-1, SP-1, cAMP-responsive element binding protein, and CEBP) by using the Genomatrix[7] and TFSEARCH[8] programs. These computational algorithms identified a total of 70 unique ERα promoter targets, which were used to construct a subpanel genomic microarray (see a partial list of the genes in Supplementary Table S4). The previously described DMH method (25, 26) was then used to determine the DNA methylation status of these ERα targets in siRNA-treated *versus* mock-treated MCF-7 cells. Amplicons representing genomic pools of methylated DNAs were prepared from these treated cells using our established protocols (25, 26). Cy5 (red dye)- and Cy3 (green dye)-labeled DNAs were prepared from siRNA- and mock-treated cells, respectively, and cohybridized to microscope slides containing the arrayed 70 unique ERα targets. ERα target loci methylated in siRNA-treated cells, but not in mock-treated cells, were expected to show greater Cy5/Cy3 hybridization signals. This is because methylated CpG sites are protected from methylation-sensitive restriction (*i.e.*, *Hpa*II and *Bst*UI) and could thus be amplified by a linker-PCR approach during amplicon preparation. In contrast, unmethylated CpG sites were restricted by the methylation-sensitive enzymes, could not be amplified by PCR, and were thus devoid of hybridization signals.

To analyze our microarray data, we adapted the "shrunken centroids method" (38) to define the threshold setting for class prediction of methylated ERα target loci. This approach can be used to uniquely define the threshold level that statistically discriminates ERα loci commonly methylated in siRNA-treated cells from the same loci in mock-treated cells. After initial evaluation of the microarray data, we chose the threshold value 2.0 that generates less error (≤0.3) for cross-validation (data not shown). When the cross-validation variances from individual samples were plotted (Fig. 5*A*), many ERα target loci could be used to discriminate between siRNA-treated cells and mock-treated counterparts (manifested as having many loci with no misclassification error) at the 168 hour time point. However, this threshold level was not sufficiently stringent to discriminate between the mock- and siRNA-treated cell samples at 24 or 36 hours (manifested as having very few loci with low misclassification error). In Fig. 5*B*, the actual methylation status of individual loci, in comparison with the predicted centroids, is plotted to present an overall change of DNA methylation at different time periods of siRNA treatment. Relative to the overall predicted centroids, a positive value of a locus

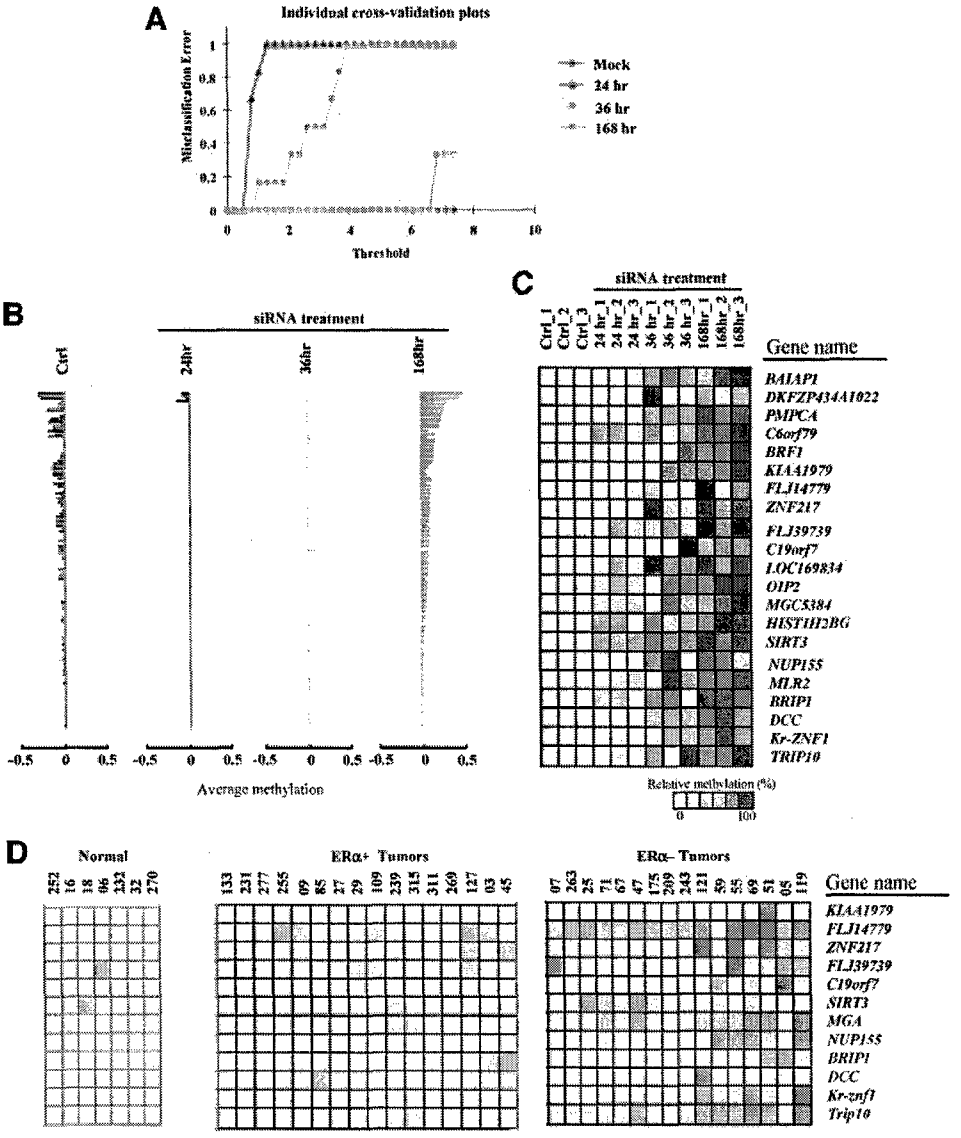

Fig. 5. Acquired DNA methylation in multiple *ERα* downstream targets after estrogen signal disruption. Seventy ERα downstream targets were analyzed by DMH, as described in the text. Fluorescence-labeled methylation amplicons were prepared from siRNA-treated (24, 36, and 168 hours) and mock-treated (168 hours) MCF-7 cells, respectively, and cohybridized to ERα microarray slides. The hybridization output is the measured relative intensity of fluorescence reporter molecules. *A*, test error for different values of shrinkage. Shrunken centroids analysis was conducted using methylation microarray datasets (see detailed description in the text). Tenfold cross-validation was used to estimate the error rate, when a different degree of shrinkage was used to generate the centroids. *B*. Predicted centroids, shown as horizontal units, represent log ratios of DNA methylation. The order of the 70 ERα target loci is arbitrary. Methylation changes were seen only in a few loci at 24 or 36 hours after siRNA treatment; however, a significant methylation change was seen at 168 hours (7 days; $P < 0.05$), displaying positively shrunken values for these 70 loci. *C*, methylation heat map of the 21 selected ERα loci at different time periods after siRNA treatment. These loci were selected because a threshold (threshold = 2; error rate ≤ 0.3) from cross-validation showed fewer errors in methylation microarray experiments. As shown, DNA methylation of these loci accumulates progressively over time (168 hours) after the siRNA treatment. Data shown here represent three independent microarray experiments. *D*, methylation heat map of the top 12 methylated ERα loci in ERα-negative tumors. Microarray-based DMH was conducted in these clinical samples as described in the text. The derived microarray data were analyzed by the shrunken centroids method.

indicates more methylation during the treatment, whereas a negative value indicates less methylation. This shrunken centroids map revealed that *de novo* DNA methylation can be detected in a subset of ERα targets 168 hours after siRNA treatment, but not in cells treated for only 24 or 36 hours after treatment.

To validate the findings of the shrunken centroid analysis, unsupervised cluster analysis was performed on the microarray data, using the top 21 methylated loci selected by machine training ("heat map" shown in Fig. 5*C*). The result reaffirms the shrunken centroid data in that replicates of each treatment type are clustered together and that the level of methylation increased with the extent of siRNA treatment. A paired *t* test revealed that the methylation status of these 21 loci was significantly different ($P < 0.05$) between the mock-treated (ERα-positive) and siRNA-treated (ERα-negative) cells.

This microarray observation was independently validated by conducting expression and DNA methylation analyses on three newly identified ERα downstream targets, *TRIP10*, *Kr-Znf1*, and *DCC*. In general, the decreased levels of these mRNAs preceded the emergence of DNA methylation at their respective promoter CpG islands (Fig. 6*A* and *B*). This epigenetically mediated silencing also indirectly influenced the expression of *MTA3*, a gene known to be regulated via a downstream ERα target and to participate in Mi-2/NuRD nucleosome remodeling (Fig. 6*B*; ref. 39).

DNA Methylation of ERα Downstream Targets is Preferentially Observed in ERα-Negative Tumors. We next determined whether this *in vitro* finding could be seen *in vivo*. DMH was therefore conducted using the aforementioned 32 primary breast tumors and 7 normal controls. The derived microarray data were then analyzed by the shrunken centroid method. Although the methylation results of these 70 ERα target loci did not clearly segregate tumor samples into subclasses, we observed a general trend that methylated loci appear more frequently in ERα-negative tumors than in ERα-positive tumors ($P < 0.05$). Fig. 5*D* presents a heat map of the 12 most methylated loci in the studied breast tumors. As shown, we observed higher overall methylation in the ERα-negative tumors (6 of 16 tumors had >40% methylation in the loci analyzed) than in the ERα-positive tumors (only 1 of 16 tumors achieved the same level of methylation). Also, the total number of loci showing DNA methylation was greater in ERα-negative tumors, when compared with ERα-positive tumors. Only four loci showed a low level of methylation in normal breast samples. Methylation analysis by MSP was further conducted for *TRIP10* in these breast samples (Fig. 6*C*). Consistent with the microarray finding, *TRIP10* promoter hypermethylation was detected in 50% (8 of 16) of ERα-negative tumors but in none of the 16 ERα-positive tumors analyzed (χ^2 test, $P < 0.005$).

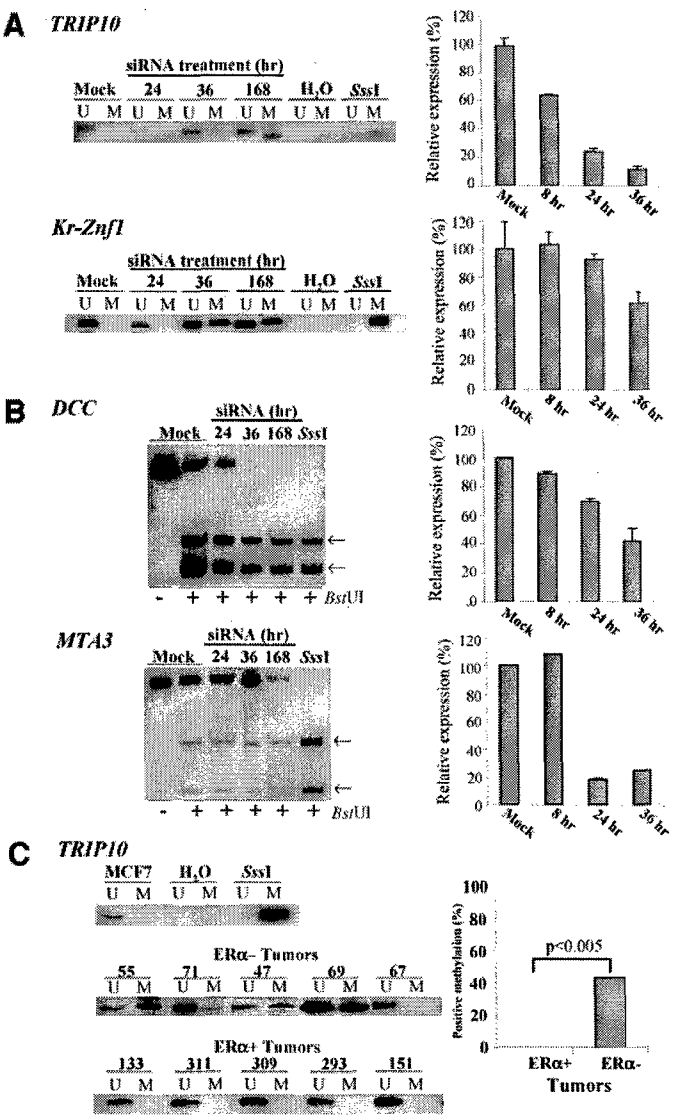

Fig. 6. Methylation and expression analysis of *ERα* target genes. *A*. For methylation-specific PCR assays, bisulfite-treated DNA samples from siRNA- or mock-treated cells were used for amplification with specific primers for *TRIP10* and *Kr-Znf1*, respectively. ^{32}P-labeled PCR products for unmethylated (*Lanes U*) and methylated (*Lanes M*) DNA strands were separated and displayed on 6% polyacrylamide gels (*left panels*). Messenger RNA levels of these genes were measured by quantitative real-time RT-PCR (*right panels*) and normalized using *GAPDH* mRNA expression levels, as described in the text. Results from three independent experiments are shown as means ± SE. The expression level of these genes was significantly reduced by the siRNA treatment (paired *t* test, $P < 0.017$ for *TRIP10* and $P < 0.048$ for *Kr-Znf1*). *B*. For *DCC* and *MTA* genes, COBRA was used to measure DNA methylation. Bisulfite DNA samples were amplified and digested with *Bst*UI enzyme and separated on polyacrylamide gels. The digested DNA fragments shown by *arrows* reflect methylation at the *Bst*UI restriction sites within the *DCC* promoter CpG island (*left panels*). Messenger RNA levels of these genes were measured by quantitative real-time RT-PCR (*right panels*) and normalized using *GAPDH* mRNA expression levels, as described in the text. Results from three independent experiments are shown as means ± SE. The expression level of these genes was significantly reduced by siRNA treatment (paired *t* test, $P < 0.03$ for *DCC* and $P < 0.014$ for *MTA3*). *C*. MSP analysis of the *TRIP10* promoters in 16 ERα-positive and 16 ERα-negative breast tumors. Only representative results are shown.

DISCUSSION

Understanding the sequence of how complex epigenetic events are established can provide important insights into the molecular mechanisms underlying gene silencing in cancer. However, the "chicken and egg" issue of which comes first, DNA methylation, histone modification, or others, is an ongoing debate in the epigenetic research community. Many early studies of this issue come from non-mammalian systems. Mutations in a histone methyltransferase specific for H3-K9 resulted in loss of DNA methylation in *Neurospora crassa* (15, 16), suggesting that histone methylation can initiate DNA methylation. In *Arabidopsis*, it has been shown that CpNpG methylation depends on a histone H3 methyltransferase (40), also indicating that histone methylation can direct DNA methylation. New evidence suggests that the reverse scenario can occur in heterochromatin (41). In this case, a self-reinforcing system is implemented, allowing for feedback from DNA methylation to histone methylation for the long-term maintenance of a heterochromatin state in a gene (41). However, this epigenetic paradigm remains to be explored in mammalian systems. Earlier studies have shown that *in vitro* methylated transgenes can be targets for methyl-CpG–binding proteins, which in turn recruit repressor complexes containing histone deacetylases (17, 18). Fahrner *et al.* (19) suggested that DNA methylation of *hMLH1* can specify unique histone codes for the maintenance of a silenced state. They detected methyl histone 3-lysine 9 in the DNA methylated, transcrip-

tionally silenced promoter CpG island of *hMLH1* in a cancer cell line. Treatment with the DNA demethylating agent 5-AzadC alone, but with not the histone deacetylase inhibitor trichostatin A, resulted in reversal of this repressive histone modification. Taken together, these reports, as well as other studies, imply that in contrast to other organisms, histone modifications may be secondary to DNA methylation in initiating gene silencing in mammalian cells (17, 18, 20, 42).

A study by Bachman *et al.* (43), however, presents a different view with respect to the silencing of the *p16* gene in an experimental system using somatic knockout cells. These authors suggest that chromatin modifications are not totally dependent on prior DNA methylation to initiate gene silencing. In support of this observation, Mutskov and Felsenfeld (44) have recently demonstrated that histone modifications are the primary event associated with the silencing of a transgene, *ILR2*. In this case, a gradual increase in DNA methylation density in and around the *ILR2* promoter was observed after transfection. In contrast to previous observations, these two recent studies therefore suggest that DNA methylation sets up an epigenetic "mark" for the maintenance of long-term silencing, rather than initiating it. Clearly, this epigenetic process is complex and multifaceted, and it is possible that the sequence of epigenetic events for establishing and maintaining the silenced state of a gene can be locus or pathway specific.

The present study suggests that gene inactivation and histone modifications occur before DNA methylation at some ERα target loci. Depicted in Fig. 7 is a hypothetical gene containing an ERE site within the promoter area, the active transcription of which is directly dependent on estrogen signaling. On the removal of this signaling, down-regulation of this gene occurs immediately. Transcriptional repressors (*e.g.*, polycomb proteins) and histone deacetylases are then assembled to its promoter to initiate long-term transcriptional repression. Subsequent recruitment of DNA methyltransferases to the repressor complex methylates CpG sites in the adjacent area. This process may be gradual, with methylation density increasing over time in the targeted area (see the heat map in Fig. 5*C*). The buildup of DNA methylation could set up a heritable mark that may eventually replace some of the original repressors to establish a heterochromatin state of long-term silencing. In this case, reactivation of ERα target genes could no longer be achieved by reestablishing estrogen signaling alone (see the example of *PR* in Fig. 4*A*); it also requires DNA demethylation. In addition to the *PR* gene, we suggest that establishment of epigenetic memory may occur in other critical ERα downstream loci in some breast cancer cells.

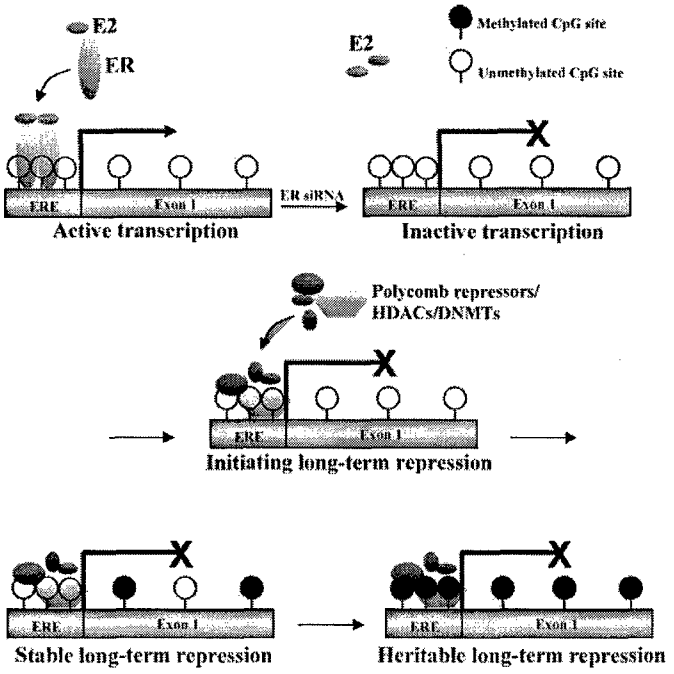

Fig. 7. A proposed model for the epigenetic hierarchy of long-term silencing in *ERα* downstream targets (see further explanation in the text). *HDACs*, histone deacetylases; *DNMTs*, DNA methyltransferases.

The occurrence of DNA methylation in a pathway-specific manner also has a new implication. Altered DNA methylation was originally thought to be a generalized phenomenon arising from a stochastic process in earlier studies (45, 46). This random methylation in tumor suppressor genes at their promoter CpG islands, thus silencing their transcripts, would provide tumor cells with a growth advantage. The specific epigenetic patterns observed in particular cancer types would therefore be derived from clonal selection of the proliferating cells. Some studies (26, 47, 48), however, have indicated that this epigenetic event is not random and that remodeling of the local chromatin structure of a gene may influence its susceptibility to specific DNA methylation. The present study provides some answers to this conundrum. Here we show that dysregulation of normal signaling in cancer cells may result in stable silencing of downstream targets, maintained by epigenetic machinery. This implies that the altered epigenetic condition is pathway specific, rather than a stochastic process in the ERα signaling pathway.

In conclusion, the present study implicates, for the first time, epigenetic influence (*i.e.*, chromatin remodeling and DNA methylation) on transcription of ERα downstream target genes and thus provides a new direction for research in this classical signaling pathway. Unlike irreversible genetic damage, epigenetic alterations are potentially reversible, providing an opportunity for therapeutic intervention in breast cancer. Histone deacetylase inhibitors, alone or together with DNA demethylating agents, may represent novel treatment approaches that could be combined with currently available chemotherapies. Our experimental evidence therefore provides a rationale for such treatment strategies designed to alter aberrant epigenetic processes in hormone-insensitive but receptor-positive breast tumors.

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